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DYSREGULATION OF TUMOUR NECROSIS FACTOR IN THE BB RAT MODEL OF HUMAN INSULIN-DEPENDENT DIABETES MELLITUS

by

PETER H. LAPCHAK

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

in the

DEPARTMENT OF MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

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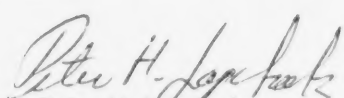

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DATE: September 9, 1994

THE GENTLE GIANTS

There are the gentle giants
Who walk softly through our lives.

No shrill demand for early recognition
Commands our attentive admiration.

No blinding glare of brilliance
Announces their presence amongst us.

They are the quietly courageous ones
Who plant for future generations.

They are the creators and searchers
Who teach and celebrate the truth.

. . . taken from Current Contents, circa 1984, author unknown.

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **DYSREGULATION OF TUMOUR NECROSIS FACTOR IN THE BB RAT MODEL OF HUMAN INSULIN-DEPENDENT DIABETES MELLITUS** submitted by **PETER H. LAPCHAK** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **MEDICAL SCIENCES (MEDICINE)**.

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To Janet, for giving me strength, support and unconditional love; and ...

To my parents, for their love, support, and encouragement from across the miles.

ABSTRACT

Tumour necrosis factor-alpha (TNF-α) is implicated as a mediator of autoimmune diabetes although TNF-α or complete Freund's adjuvant (CFA) treatment prevents diabetes in diabetes-prone (DP) BB rats. Protection by CFA or TNF-α may increase TNF to the necessary threshold levels suggesting that a deficiency in TNF-α may predispose BB rats to develop diabetes. These hypotheses were tested in experiments where TNF levels were measured in lymphoid cells populations from untreated and CFA-treated DP rats and in experiments which further characterized the nature of this deficiency.

Tumour necrosis factor levels by DP rat peritoneal cells and thymocytes are **deficient** after stimulation *in vivo* or *in vitro* compared to DR and old non-diabetic DP rats. After treatment with CFA, peritoneal cell TNF levels were **increased**. In contrast, DP rat spleen cell TNF levels are **increased** and were **reduced** after CFA treatment as were DP rat serum TNF levels. DP rat serum corticosterone levels were decreased compared to DR rats. One mechanism by which CFA may prevent diabetes is to **increase** peritoneal cell, and **decrease** spleen cell and serum TNF levels. Long-term administration of a polyclonal antisera to TNF **reversed** CFA-mediated protection against diabetes in DP rats.

Deficient or dysregulated TNF levels by DP rat peritoneal cells suggests deficiencies or defects in lipopolysaccharide (LPS) and CD14 receptor expression. No deficiency in expression of either receptor was observed. Other mechanisms of macrophage TNF production were evaluated to determine whether defects were present in other receptors or signal transduction pathways. Tumour necrosis factor production was **deficient** in response to interleukin-2 (IL-2), IFN-γ, and LPS, and in response to protein kinases and/or Ca^{2+} agonists suggesting that defects in TNF release may lie distal to defects in signal transduction pathways. Alternatively, TNF production may be suppressed. TNF levels were **decreased** and prostaglandin E_2 (PGE_2) levels were **increased** from DP rat peritoneal cells. In contrast, TNF levels were **increased** from DP rat peritoneal cells cultured with a prostaglandin inhibitor, indomethacin, and PGE_2 levels were **decreased**, similar to CFA-treated DP rats. In conclusion, TNF production by DP rat lymphoid cells is dysregulated and is corrected after CFA treatment. Administration of anti-TNF anti-sera reverses this correction. Over-production of, or hypersensitivity to PGE_2, by DP rat peritoneal cells may result in suppression of TNF production. Complete Freund's adjuvant may protect against diabetes by **increasing** peritoneal cell TNF and **decreasing** PGE_2.

RESUMÉ

Le facteur de nécrose des alpha (TNF-α) est un médiateur des diabètes auto-immuns, bien que la thérapie par le TNF-α ou l'adjuvant complet de Freund (CFA) empêche le développement du diabète chez des rats BB qui y sont prédisposés. L'effet protecteur du TNF-α ou du CFA doit être médié par une élévation du taux de TNF-α au-delà du seuil limite, ce qui suggère qu'un déficit en TNF-α prédisposerait des rats BB a développer un diabète. Cette hypothèse a été testée à l'aide d'expériences ou le taux TNF-α a éte mesuré dans des cellules lymphoïdes extraites de rats sujets au diabete traités ou non par le CFA. D'autres expériences ont ete effectuées pour caractériser plus en détail la nature de la carence.

Le taux de TNF-α dans les thymocytes et les cellules du péritoine de rats sujets au diabète est faible après stimulation *in vivo* ou *in vitro*, si l'on compare avec celui des même cellules extraites de rats DR ou de rats sujets au diabète mais ne l'ayant pas développé depuis longtemps. Le taux de TNF-α dans les cellules du péritoine est accru par la thérapie au CFA. Au contraire, le taux de TNF-α dans les cellules de rate et dans le sérum des rats sujets au diabète est augmenté après stimulation et diminué par la thérapie au CFA. Le taux de corticostérone dans le sérum des rats sujets au diabète est plus faible que pour le rats DR. Un des mécanismes par lequel le CFA empêcherait le développement du diabète serait d'augmenter le taux de TNF-α dams les cellules du péritoine et de le diminuer dans les cellules de rate et le sérum. L'administration continue d'anticorps anti-TNF empêche l'effet protecteur du CFA chez les rats sujets au diabète.

Le carence en TNF-α dms les cellules de péritoine de rats sujets au diabète suggère que l'expression des récepteurs du lipopolysaccharide (LPS) ou du CD14 serait affectée. Aucune différence dans l'expression de ces recepteurs n'a été observée. D'autres mécanismes de production du TNF-α par les macrophage sont été étudiés pour déterminer si d'autres récepteurs ou les voies de transduction des signaux ne seraient pas affecteés. La production de TNF-α est déficiente en réponse à l'interleukine 2 (IL-2), l'interferon gamma (l'IFN-γ), le LPS, les proteines kinases et/ou les agonistes du Ca^{2+}, ce qui suggère que la carence en TNF-α serait dûe à une déficience localisée en aval des voies de transduction des signaux. Eventuellement, la production de TNF-α peut être abolie. Le taux de TNF-α est diminué et le taux de prostaglandine E_2 (PGE_2) augmenté dans les cellules du péritoine de rats sujets au diabète. Au contraire, le taux de TNF-α est augmenté dans les cellules de péritoine de rats sujets au diabète quand elles sont cultivées en présence d'un inhibiteur de la prostaglandine, l'indométhacine, alors que le taux de PGE_2 est diminué, comme pour les rats sujets au diabète traités par le CFA. En conclusion, la production de TNF-α par les cellules lymphoïdes de rats sujets au diabète est dérégulée, et ceci peut être réparé par la thérapie au CFA. L'administration continue d'anticorps anti-TNF supprime l'effet correcteur du CFA. La surproduction de PGE_2 ou une hypersensibilité au PGE_2 par les cellules de péritoine de rats sujets au diabète pourrait provoquer l'arrêt de la production de TNF-α. L'adjuvant complet de Freund protegerait contre le diabète en augmentant le taux de TNF-α des cellules du péritoine et en diminuant le taux de PGE_2.

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TABLE OF CONTENTS

	PAGE
CHAPTER ONE: *INTRODUCTION*	1
– *THE BB RAT: ORIGINS, HISTORY AND CLINICAL FEATURES*	1
– *PATHOLOGY: THE DIABETIC PANCREAS*	1
– *GENETICS AND THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ASSOCIATION WITH DIABETES IN BB RATS*	1
· THE RAT MHC	
· BREEDING STUDIES	
· MOLECULAR ANALYSIS OF MHC GENE POLYMORPHISMS IN BB RATS	
– *AUTOANTIBODIES IN THE BB RAT*	2
– *LYMPHOCYTE POPULATION AND FUNCTION IN THE BB RAT*	2
· LYMPHOPENIA	
· RT6$^+$ T LYMPHOCYTES AND DIABETES	
· HELPER T (T_h) LYMPHOCYTE SUBSETS IN THE BB RAT	
· MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ANTIGEN EXPRESSION ON BB RAT LYMPHOCYTES	
· RESPONSES *IN VITRO* BY BB RAT T LYMPHOCYTES	
· NATURAL KILLER (NK) CELLS IN THE BB RAT	
· ISLET CELL-SPECIFIC T LYMPHOCYTES IN THE BB RAT	
– *MACROPHAGES AND DENDRITIC CELLS IN THE BB RAT*	7
– *IMMUNE-MEDIATED ISLET CELL KILLING*	8
· COMPLEMENT-DEPENDENT ANTIBODY-MEDIATED ISLET CELL KILLING	
· MACROPHAGE-MEDIATED ISLET CELL KILLING	
· NATURAL KILLER (NK) CELL-MEDIATED ISLET CELL KILLING	
– *PASSIVE TRANSFER OF DIABETES*	9
– *CYTOKINES AND ISLET CELL KILLING*	10
– *MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PANCREATIC ISLET β CELLS*	13
– *PREVENTION OF DIABETES IN THE BB RAT: IMMUNOSUPPRESSION*	14
· BONE MARROW TRANSPLANTATION	
· NEONATAL THYMECTOMY AND THYMIC TRANSPLANTATION	
· LYMPHOCYTE TRANSFUSION	
· ANTIBODY-MEDIATED PREVENTION	
· PHARMACOLOGICAL PREVENTION OF DIABETES	
· VIRUSES	
· OTHER METHODS	
– *PREVENTION OF DIABETES: IMMUNOREGULATION*	18
· MITOGENS AND DIABETES	
· ADJUVANTS AND DIABETES	
· CYTOKINES AND DIABETES	
– *TUMOUR NECROSIS FACTOR AND DIABETES*	23
· TUMOUR NECROSIS FACTOR PROTEINS	
· IMMUNOLOGY OF TUMOUR NECROSIS FACTOR	
· ADMINISTRATION OF TUMOUR NECROSIS FACTOR	

- TUMOUR NECROSIS FACTOR GENE POLYMORPHISMS AND DIABETES
- TUMOUR NECROSIS FACTOR TRANSGENIC ANIMALS

– *STATEMENT OF THE PROBLEM* 27

CHAPTER 2: *MATERIALS AND METHODS* 28

- ANIMALS 28
- TREATMENT WITH COMPLETE FREUND'S ADJUVANT 28
- PREPARATION OF CELLS 28
- *IN VITRO* ACTIVATION OF CELLS 29
- PREPARATION OF CELLULAR COMPONENTS 29
- STIMULATION OF RATS BY INJECTION *IN VIVO* 30
- TUMOUR NECROSIS FACTOR (TNF) ASSAY 30
- INDUCTION OF TOLERANCE TO IMMUNOGLOBULINS (Ig) IN NEONATAL RATS 31
- QUANTITATION OF RABBIT IMMUNOGLOBULINS (Ig) BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) 31
- PRODUCTION OF RABBIT ANTI-MURINE TUMOUR NECROSIS FACTOR-ALPHA (TNF-α) POLYCLONAL ANTISERA 32
- DETERMINATION OF TNF-α NEUTRALIZING ACTIVITY IN RABBIT SERA 32
- AMMONIUM SULPHATE PRECIPITATION OF IMMUNOGLOBULINS (Ig) FROM RABBIT SERA 33
- DETECTION OF RABBIT ANTI-TNF IMMUNOGLOBULINS IN $(NH_4)_2SO_4$-PRECIPITATED RABBIT SERA AND IN RAT SERA BY ELISA 33
- TREATMENT OF BB RATS WITH RECOMBINANT TUMOUR NECROSIS FACTOR-α 34
- TREATMENT OF RATS WITH POLYCLONAL ANTISERUM TO TUMOUR NECROSIS FACTOR-α 34
- RADIOIMMUNOASSAY OF PROSTAGLANDIN E_2 (PGE_2) 35
- RADIOIMMUNOASSAY OF SERUM CORTICOSTERONE 35
- TWO-COLOUR STAINING OF PERITONEAL CELLS AND FLUORESCENT ACTIVATED CELL SORTING (FACS) ANALYSIS 37
- INDIRECT DETECTION OF LIPOPOLYSACCHARIDE RECEPTORS ON PERITONEAL CELLS 37
- STATISTICAL ANALYSIS 38

RESULTS

CHAPTER 3: *TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE BB RATS IS DEFICIENT AND CAN BE CORRECTED BY TREATMENT WITH COMPLETE FREUND'S ADJUVANT* 39

- INTRODUCTION 39
- RESULTS 39
- DISCUSSION 41
- SUMMARY 42
- TABLES 43
- FIGURES 45

CHAPTER 4: *EXCESSIVE TUMOUR NECROSIS FACTOR BY SPLEEN CELLS FROM DIABETES-PRONE BB RATS IS DECREASED BY TREATMENT* IN VIVO *WITH COMPLETE FREUND'S ADJUVANT* 49
- INTRODUCTION 49
- RESULTS 50
- DISCUSSION 51
- SUMMARY 52
- TABLES 53
- FIGURES 55

CHAPTER 5: *DYSREGULATION OF TUMOUR NECROSIS FACTOR PRODUCTION IS TISSUE-SPECIFIC AND CORRECTED AFTER TREATMENT WITH COMPLETE FREUND'S ADJUVANT* 59
- INTRODUCTION 59
- RESULTS 59
- DISCUSSION 61
- SUMMARY 62
- TABLES 63
- FIGURES 64

CHAPTER 6: *TUMOUR NECROSIS FACTOR PRODUCTION BY LYMPHOID CELLS FROM YOUNG DIABETES-PRONE RAT DIFFER FROM OLD NON-DIABETIC DIABETES-PRONE BB RATS* 67
- INTRODUCTION 67
- RESULTS 67
- DISCUSSION 70
- SUMMARY 72
- TABLES 73
- FIGURES 79

CHAPTER 7: *SERUM TUMOUR NECROSIS FACTOR RESPONSES ARE INCREASED IN DIABETES-PRONE BB RATS: A POSSIBLE RELATIONSHIP TO DEFICIENT CORTICOSTERONE RESPONSES* 84
- INTRODUCTION 84
- RESULTS 85
- DISCUSSION 86
- SUMMARY 88
- FIGURES 89

CHAPTER 8: *POLYCLONAL ANTISERUM TO TUMOUR NECROSIS FACTOR REVERSES COMPLETE FREUND'S ADJUVANT-MEDIATED PROTECTION AGAINST DIABETES IN DIABETES-PRONE BB RATS* 95
- INTRODUCTION 95
- RESULTS 95
- DISCUSSION 96
- SUMMARY 98
- TABLES 99
- FIGURES 102

CHAPTER 9: *DEFICIENT TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE BB RATS IS NOT DUE TO THE ABSENCE OF LIPOPOLYSACCHARIDE RECEPTORS* 106
- INTRODUCTION 106
- RESULTS 106
- DISCUSSION 108
- SUMMARY 109
- TABLES 110
- FIGURES 115

CHAPTER 10: *ALTERNATE MECHANISMS OF MACROPHAGE ACTIVATION FAIL TO STIMULATE DIABETES-PRONE BB RAT PERITONEAL CELL TUMOUR NECROSIS FACTOR (TNF) PRODUCTION: CORRECTION BY INDOMETHACIN OR COMPLETE FREUND'S ADJUVANT* 120
- INTRODUCTION 120
- RESULTS 121
- DISCUSSION 122
- SUMMARY 124
- FIGURES 125

CHAPTER 11: *MODULATION OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX CELL SURFACE ANTIGEN EXPRESSION ON BB RAT PERITONEAL CELLS AFTER TREATMENT WITH COMPLETE FREUND'S ADJUVANT* 130
- INTRODUCTION 130
- RESULTS 130
- DISCUSSION 131
- SUMMARY 132
- TABLES 133
- FIGURES 138

CHAPTER 12: *DISCUSSION AND SUMMARY* 141
– *DISCUSSION* 141
– *SUMMARY* 144

CHAPTER 13: *BIBLIOGRAPHY* 146

LIST OF TABLES

TABLE 3.1:	*TNF PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS*	43
TABLE 3.2:	*EFFECTS OF TREATMENT OF BB RATS WITH COMPLETE FREUND'S ADJUVANT (CFA) ON TNF PRODUCTION BY PERITONEAL CELLS*	44
TABLE 4.1:	*TNF PRODUCTION BY SPLEEN CELLS OF DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS*	53
TABLE 4.2:	*EFFECTS OF TREATMENT ON BB RATS WITH COMPLETE FREUND'S ADJUVANT (CFA) ON TNF PRODUCTION BY SPLEEN CELLS*	54
TABLE 5.1:	*EFFECTS OF TREATMENT OF BB RATS WITH COMPLETE FREUND'S ADJUVANT (CFA) ON UNSTIMULATED CYTOSOLIC TNF BY DIFFERENT CELL TYPES*	63
TABLE 6.1:	*TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES*	73
TABLE 6.2:	*TNF LEVELS IN UNSTIMULATED SPLEEN CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES*	74
TABLE 6.3:	*TNF LEVELS IN UNSTIMULATED THYMOCYTES FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES*	75
TABLE 6.4:	*TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIFFERENT RAT STRAINS*	76
TABLE 6.5:	*TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIFFERENT RAT STRAINS AFTER CULTURE* IN VITRO	77
TABLE 6.6:	*SERUM TNF LEVELS FROM DIFFERENT RAT STRAINS (UNSTIMULATED)*	78
TABLE 8.1:	*NEUTRALIZATION OF MURINE TNF-α ACTIVITY ON L929-8 CELLS BY RABBIT SERUM*	99
TABLE 8.2:	*DETECTION OF ANTIBODIES TO MURINE TNF-α IN RABBIT ANTISERUM AND IN SERA OF DP RATS TREATED WITH ANTISERUM TO MURINE TNF-α*	100

TABLE 8.3:	*NEUTRALIZATION OF MURINE TNF-α ACTIVITY AGAINST L929-8 CELLS BY SERA OF DP RATS TREATED WITH RABBIT ANTISERUM TO MURINE TNF-α*	101
TABLE 9.1:	*FLOW CYTOMETRIC DETERMINATION OF PERITONEAL CELLS FROM BB RATS*	110
TABLE 9.2:	*TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF LIPOPOLYSACCHARIDE RECEPTOR (LPS-R)-POSITIVE CELLS FROM BB RATS*	111
TABLE 9.3:	*TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF LIPOPOLYSACCHARIDE RECEPTOR (LPS-R) CD14-POSITIVE CELLS FROM BB RATS*	112
TABLE 9.4:	*FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR THE LIPOPOLYSACCHARIDE RECEPTOR (LPS-R) AND CD14*	113
TABLE 9.5:	*PEAK CHANNEL FLUORESCENCE OF LIPOPOLYSACCHARIDE RECEPTOR (LPS-R) AND CD14 ON BB RAT PERITONEAL CELLS*	114
TABLE 11.1:	*TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PERITONEAL CELLS FROM BB RATS*	133
TABLE 11.2:	*TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PERITONEAL CELLS FROM FROM BB RATS*	134
TABLE 11.3:	*FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION*	135
TABLE 11.4:	*FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION*	136
TABLE 11.5:	*PEAK CHANNEL FLUORESCENCE OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS ON ON BB RAT PERITONEAL CELLS*	137

LIST OF FIGURES

FIGURE 3.1: *TNF PRODUCTION BY PERITONEAL CELLS FROM DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS STIMULATED* IN VIVO 45

FIGURE 3.2: *TNF PRODUCTION BY PERITONEAL CELLS FROM DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS STIMULATED* IN VITRO 46

FIGURE 3.3: *TNF PRODUCTION BY PERITONEAL CELLS FROM COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS STIMULATED* IN VITRO 47

FIGURE 3.4: *NEUTRALIZATION OF RAT PERITONEAL CELL TNF-MEDIATED CYTOTOXICITY OF L929-8 CELLS BY A RABBIT POLYCLONAL ANTIBODY TO MURINE TNF-α* 48

FIGURE 4.1: *TNF PRODUCTION BY SPLEEN CELLS FROM DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS RATS STIMULATED* IN VIVO 49

FIGURE 4.2: *TNF PRODUCTION BY SPLEEN CELLS FROM DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS STIMULATED* IN VIVO 50

FIGURE 4.3: *TNF PRODUCTION BY SPLEEN CELLS FROM DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS STIMULATED* IN VITRO 51

FIGURE 4.4: *TNF PRODUCTION BY SPLEEN CELLS FROM COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DIABETES-RESISTANT (DR) AND DIABETES-PRONE (DP) RATS RATS STIMULATED* IN VITRO 52

FIGURE 5.1: *TUMOUR NECROSIS FACTOR (TNF) PRODUCTION BY PERITONEAL CELLS FROM SALINE- OR COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) RATS STIMULATED* IN VITRO 64

FIGURE 5.2: *TUMOUR NECROSIS FACTOR (TNF) PRODUCTION BY SPLEEN CELLS FROM SALINE- OR COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) RATS STIMULATED* IN VITRO 65

FIGURE 5.3: *TUMOUR NECROSIS FACTOR (TNF) PRODUCTION BY THYMOCYTES FROM SALINE- OR COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) RATS STIMULATED* IN VITRO 66

FIGURE 6.1: *TNF PRODUCTION BY PERITONEAL CELLS FROM YOUNG AND OLD DR AND DP RATS AFTER STIMULATION* IN VITRO 78

FIGURE 6.2: *TNF PRODUCTION BY SPLEEN CELLS FROM YOUNG AND OLD DR AND DP RATS AFTER STIMULATION* IN VITRO 79

FIGURE 6.3: *PEAK TNF PRODUCTION BY PERITONEAL CELLS FROM YOUNG AND OLD DR AND DP RATS AFTER STIMULATION* IN VITRO 80

FIGURE 6.4: *TNF PRODUCTION BY SPLEEN CELLS FROM YOUNG AND OLD DR AND DP RATS AFTER STIMULATION* IN VITRO 81

FIGURE 6.5: *TNF PRODUCTION BY THYMOCYTES FROM YOUNG AND OLD DR AND DP RATS AFTER STIMULATION* IN VITRO 82

FIGURE 7.1: *SERUM TNF LEVELS IN DIABETES-RESISTANT (DR)* AND DIABETES-PRONE (DP) RATS STIMULATED IN VIVO 89

FIGURE 7.2: *SERUM TNF AND CORTICOSTERONE LEVELS IN DIABETES-RESISTANT (DR), DIABETES-PRONE (DP), LEWIS, AND WISTAR FURTH (WF) RATS STIMULATED* IN VIVO 90

FIGURE 7.3: *SERUM TNF AND CORTICOSTERONE LEVELS IN DIABETES-RESISTANT (DR), DIABETES-PRONE (DP), LEWIS, AND WISTAR FURTH (WF) RATS STIMULATED* IN VIVO 91

FIGURE 7.4: *SERUM TNF AND CORTICOSTERONE LEVELS IN DIABETES-RESISTANT (DR), DIABETES-PRONE (DP), LEWIS, AND WISTAR FURTH (WF) RATS STIMULATED* IN VIVO 92

FIGURE 7.5: *SERUM TNF AND CORTICOSTERONE LEVELS IN DIABETES-RESISTANT (DR), DIABETES-PRONE (DP), AND COMPLETE FREUND'S ADJUVANT (CFA)-TREATED DP AND DR RATS STIMULATED* IN VIVO 93

FIGURE 7.6: *TNF PRODUCTION BY PERITONEAL CELLS FROM DIABETES-RESISTANT (DR), DIABETES-PRONE (DP), LEWIS AND WISTAR FURTH (WF) RATS STIMULATED* IN VIVO 94

FIGURE 8.1: *EFFECT OF RABBIT ANTISERUM TO MURINE TNF-α ON COMPLETE FREUND'S ADJUVANT-PROTECTED DIABETES-PRONE BB RATS* 102

FIGURE 8.2: *EFFECT OF SHEEP ANTISERUM TO MURINE TNF ON COMPLETE FREUND'S ADJUVANT-PROTECTED DIABETES-PRONE BB RATS* 103

FIGURE 8.3: *EFFECT OF TUMOUR NECROSIS FACTOR-α ON DIABETES DEVELOPMENT IN THE DIABETES-PRONE BB RAT* 104

FIGURE 8.4: *EFFECT OF SHEEP ANTISERUM TO MURINE TNF ON DIABETES-PRONE BB RATS* 105

FIGURE 9.1: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF UNSTAINED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS* 115

FIGURE 9.2: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF SECOND ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS* 116

FIGURE 9.3: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS* 117

FIGURE 9.4: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS* 118

FIGURE 9.5: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS* 119

FIGURE 10.1: *TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS AFTER STIMULATION* IN VITRO 125

FIGURE 10.2: *TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS AFTER STIMULATION* IN VITRO *WITH HELPER T, LYMPHOCYTE CYTOKINES* 126

FIGURE 10.3: *TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS AFTER DIRECT STIMULATION* IN VITRO *WITH PROTEIN KINASE AND CALCIUM CHANNEL AGONISTS* 127

FIGURE 10.4: *TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS AFTER STIMULATION* IN VITRO *WITH LIPOPOLYSACCHARIDE AND A CYCLOOXYGENASE INHIBITOR, INDOMETHACIN* 128

FIGURE 10.5: *PROSTAGLANDIN E_2 PRODUCTION BY PERITONEAL CELLS AFTER STIMULATION* IN VITRO *WITH LIPOPOLYSACCHARIDE AND A CYCLOOXYGENASE INHIBITOR, INDOMETHACIN* 129

FIGURE 11.1: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF FLOW CYTOMETRIC ANALYSIS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DP AND DR RATS* 138

FIGURE 11.2: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF FLOW CYTOMETRIC ANALYSIS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DP AND DR RATS* 139

FIGURE 11.3: *REPRESENTATIVE TWO-COLOUR DOT-PLOTS OF FLOW CYTOMETRIC ANALYSIS OF ANTIBODY-LABELLED PERITONEAL CELLS FROM YOUNG, OLD, AND COMPLETE FREUND'S ADJUVANT-TREATED DP AND DR RATS* 140

LIST OF ABBREVIATIONS

aa:	amino acid
Ab:	antibody
ABTS:	2,2'-Azino-*bis*-[3-ethylbenthiazoline-6-sulfonic acid]
ACK:	hypotonic ammonium chloride solution
ACTH:	adrenocorticotropic hormone
ADCC:	antibody-dependent cell cytotoxicity
AGM1:	asialo-ganglio-N-tetrasylceramide
allo-:	allogeneic
ALS:	anti-lymphocyte serum
APC:	antigen presenting cells
BB:	BioBreeding
BCG:	*Bacillus* Calmette-Guerin
BM:	bone marrow
BN:	Brown Norway
BSA:	bovine serum albumin
Buf:	Buffalo
Ca:	calcium
C'AMC:	complement-dependent, antibody-mediated cytotoxicity
cAMP:	cyclic adenosine monophosphate
CBG:	corticosteroid binding globulin
CFA:	complete Freund's adjuvant
cm:	centimetre
CML:	cell-mediated lympholysis
CO_2:	carbon dioxide
Con A:	concanavalin A
CsA:	cyclosporin A
CSF-1:	colony stimulating factor 1
CTL:	cytotoxic T lymphocyte
Cy:	cyclophosphamide
DC:	dendritic cells
dGua:	deoxyguanidine
dL:	deci-litre
DNA:	deoxyribonucleic acid
DOC:	deoxycorticosterone
DP:	diabetes-prone
DP-O:	old diabetes-prone rat
DP-Y:	young diabetes-prone rat
D'PBS:	Dulbecco's modified phosphate buffered saline
DR:	diabetes-resistant
DR-O:	old diabetes-resistant rat
DR-Y:	young diabetes-resistant rat
DTH:	delayed-type hypersensitivity
EAE:	experimental allergic encephalomyelitis
EC:	endothelial cells
EDTA:	ethanolamine diamine tetra-acetic acid
ELISA:	enzyme-linked immunosrobent assay
FBS:	fetal bovine serum
FITC:	fluorescein isothiocyanate

LIST OF ABBREVIATIONS CONT'D:

FL1:	fluorescence intensity 1
FL2:	fluorescence intensity 2
g:	grams
GAD:	glutamic acid decarboxylase
G-CSF:	granulocyte colony stimulating factor
GM-CSF:	granulocyte-macrophage colony stimulating factor
GP:	glycoprotein
HBSS:	Hank's balanced saline solution
HLA:	human leukocyte antigen
HPA:	hypothalamic-pituitary-adrenal axis
hr:	hour
HSLAS:	Health Sciences Laboratory Animal Services
hsp:	heat shock protein
Ia:	Class II major histocompatibility complex antigen
ICAM-1:	intercellular adhesion molecule 1
IDDM:	insulin-dependent diabetes mellitus
IFA:	incomplete Freund's adjuvant
IFN-:	interferon-
Ig:	immunoglobulin
IgG:	immunoglobulin G
IgM:	immunoglobulin M
I-HPA:	immune-hypothalamic-pituitary-adrenal axis
IkB:	inhibitor-nuclear factor kappa B
IL-:	interleukin
IMDM:	Iscove's modified Dulbecco's medium
INDO:	indomethacin
kDa:	kilodaltons
kg:	kilogram
L:	litre
LBP:	lipopolysaccharide binding protein
L-CA:	leukocyte common antigen
LCMV:	lymphocytic choriomeningitis virus
LE:	Long-Evans
LEW:	Lewis
LPS:	lipopolysaccharide
LPS-R:	lipopolysaccharide receptor
LPSw:	lipopolysaccharide; wheat flour
LT:	lymphotoxin
Lyp:	lymphopenia locus
M:	molar
mAb:	monoclonal antibody
MBP:	myelin basic protein
mCi:	milli-Curie
M-CSF:	macrophage colony stimulating factor
MHC:	major histocompatibility complex
mg:	milligram
Mg:	magnesium
mL:	millilitre

LIST OF ABBREVIATIONS CONT'D:

MLC:	mixed lymphocyte culture
MLR:	mixed lymphocyte reaction
mmol:	milli-molar
mRNA:	messenger ribonucleic acid
NF-kB:	nuclear factor-kappa B
ng:	nanogram
NGS:	normal goat serum
NK:	natural killer
NKCF:	natural killer cytotoxic factor
nm:	nanometer
NOD:	non-obese diabetic
NP:	nucleoprotein
NZB:	New Zealand Black
NZW:	New Zealand White
O.D.:	optical density
OS:	obese strain
p:	protein
PBS:	phosphate-buffered saline
PE:	phycoerythrin
pg:	picogram
PGE:	prostaglandin E
PHA:	phytohemagglutinin
PKC:	protein kinase C
PLA:	phospholipase A
PLC:	phospholipase C
PMA:	phorbol myristate acetate
poly I:C:	poly inosinic-polycytidylic acid
PWM:	pokeweed mitogen
r:	recombinant
RFLP:	restriction fragment length polymorphism
rh:	recombinant human
RIA:	radio-immunoassay
rm:	recombinant murine
rpm:	revolutions per minute
RT1:	rat major histocompatibility complex
SCID:	severe combined immune deficiency
scw:	streptococcal cell wall
S.E.M.:	standard error of the mean
sIg:	surface immunoglobulin
SMLR:	syngeneic mixed lymphocyte reaction
SRBC:	sheep red blood cells
SV40:	sarcoma virus 40
TBq:	tetra becquerel
TcR:	T lymphocyte receptor
$T_{c/s}$:	T cytotoxic/suppressor
TdT^+:	terminal deoxythymidine-positive
TF5:	thymosin factor 5
TGF-β:	transforming growth factor-beta

LIST OF ABBREVIATIONS CONT'D:

T_h:	T helper
TNF:	tumour necrosis factor
TNFR:	tumour necrosis factor receptor
U:	unit
ug:	microgram
uL:	microlitre
um:	micrometer
uM:	micromolar
unstim:	unstimulated
V_β:	beta chain variable region of T cell receptor
VAF:	viral antigen-free
VCAM-1:	vascular cell adhesion molecule 1
v/v:	volume/volume
vs.:	versus
vWF:	von Willebrand factor
WF:	Wistar Furth
w/v:	weight/volume
Wor:	Worcester

CHAPTER 1

INTRODUCTION

THE BB RAT: ORIGINS, HISTORY AND CLINICAL FEATURES

The Biobreeding (BB) rat was first developed at the Biobreeding Laboratories in Ottawa in 1974 from the outbred Wistar rat as an animal model to study human spontaneous juvenile-onset insulin-dependent diabetes mellitus (IDDM) (1, 2). Insulin-dependent diabetes mellitus in the BB rat shares many similarities of human IDDM including early age of onset (60-120 day of age), hypoinsulinemia, hyperglycaemia, ketoacidosis, weight loss, polyuria, polydipsia, glycosuria, and requirement for insulin therapy. The key clinical feature of IDDM is the presence of a lymphocytic infiltration around and within the islets of Langerhan and the resulting loss of insulin-producing beta (β)-cells (3-5).

PATHOLOGY: THE DIABETIC PANCREAS

During the development of acute IDDM in the BB rat, the islets of Langerhan undergo morphological changes characterized by a mononuclear-lymphocytic infiltrate composed predominantly of MHC Class II$^+$ (activated) helper T (T_h) lymphocytes, and to a lesser extent, cytotoxic/suppressor T ($T_{c/s}$) lymphocytes, B lymphocytes, macrophages, plasma cells, polymorphonuclear cells, and eosinophils (5-9). Islet β cells injured by these immune cells appear degranulated due to a marked reduction of pancreatic insulin (5). Initially, not all islets are involved and inflammatory lesions appear not only in the islets but also in periductal regions and within acinar tissue (10). During the latter stages of disease, islets appear small, are reduced in number, and are composed mostly of alpha (α)-cells with few, or no β-cells (5). There is also an apparent decrease in both α- and delta (Δ)-cell numbers (11). Beta cell loss is specific and is associated with a predominant T lymphocytic infiltrate with losses of α- and Δ-cells occurring possibly as bystanders indicating that β-cell destruction is a T lymphocyte-mediated autoimmune disease.

GENETICS AND THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ASSOCIATION WITH DIABETES IN BB RATS

THE RAT MHC: The MHC is a multigene family encoding cell surface glycoproteins involved in the discrimination of self and non-self. Class I MHC antigens are present on all nucleated cells, including red blood cells in rodents, while Class II MHC antigen expression is restricted to B and T lymphocytes, monocytes, macrophages, dendritic cells, and thymic cortical epithelium (12). The rat MHC, called *RT1,* is composed of Class I loci (*RT1*.A, *RT1*.E and *RT1*.C) and Class II loci (*RT1*.B, *RT1*.D, and *RT1*.H) (12, 13). *RT1*.B, *RT1*.D and *RT1*.H are analogous to the mouse H-2A and E subregions, and human HLA-DQ, DR and DP regions respectively (12).

BREEDING STUDIES: BB rats from the Ottawa colony are homologous for the *u/u* haplotype at *RT1*.A and *RT1*.B loci as determined by mixed lymphocyte culture (MLC) reactions from F_2 progeny of BB rats crossed with Wistar Furth ($RT1.^{u/u}$), ACI ($RT1.^{a/a}$) and LEWIS ($RT1.^{l/l}$) rats (3). Interstrain breeding studies suggest three components are necessary for diabetes development: the *u* haplotype or a gene closely linked with the gene encoding the *u* haplotype, a susceptibility for pancreatic lymphocytic infiltration and a susceptibility to lymphopenia (14, 15). Diabetes occurs in interstrain crosses with any Class II MHC *u* haplotype regardless of the Class I MHC haplotype indicating that only the

Class II MHC *u* haplotype is necessary for diabetes development (15-18). These results suggest that Class II *u* haplotype gene products may confer susceptibility to diabetes.

F_2 progeny from sibling F_1 crosses, but not F_1 progeny from diabetes-resistant (DR) X diabetes-prone (DP) F_1 crosses develop diabetes with a pathology similar to DP rats (19). These results suggest that diabetes and insulitis are transmitted together in a recessive manner and that lymphopenia co-segregates nearly completely with diabetes and insulitis (19). Inheritance of lymphopenia occurs through segregation of a recessively-acting locus, *Lyp*, and the inheritance of diabetes results from the segregation of *Iddm1*, *Iddm2*, and *Iddm3* loci (20). Rats that are lymphopenic, and are *RT1*^{u} at Class II MHC loci on a non-*RT1*^{u} background, do not become diabetic supporting the idea that at least three genes are necessary for diabetes development (21). More recently, the rat tumour necrosis factor alpha (TNF-α) locus was mapped to the MHC suggesting that genes within the TNF-α locus may also contribute to diabetes and insulitis (22).

MOLECULAR ANALYSIS OF MHC GENE POLYMORPHISMS IN BB RATS: Polymorphisms in BB rat Class II MHC genes are thought to associate with diabetes susceptibility. Restriction fragment length polymorphism (RFLP) analysis of BB and BB-derived rats, and WF rats revealed no polymorphisms in the these genes (23-25). This was confirmed by one- and two-dimensional electrophoresis of Class II MHC proteins (26). No *RT1*.B and *RT1*.D β-chain sequence differences exist in DP- and DR-BB, and WF rats (27, 28). Class II MHC genes and their products, while not polymorphic, in combination with other factors may be sufficient to confer susceptibility to, and development of diabetes in the BB rat.

AUTOANTIBODIES IN THE BB RAT

In BB rats, numerous autoantibodies appear early and react with islet cell surface antigens, spleen and blood lymphocytes, gastric parietal cells, skeletal and smooth muscle, nuclear protein, thyroid colloid, and insulin suggesting that they may participate in the diabetic process (29-32). Islet cell surface antibodies are found in both low- and high-incidence BB rats even in the absence of insulitis or diabetes development (33). Antibodies to islet cell and $CD4^+$ and $CD8^+$ lymphocyte surface antigens generally precede the development of insulitis and diabetes in the BB rat (34, 35). The presence of these autoantibodies may contribute to the diabetic process in BB rats and also suggests that humoral immune defects may exist.

A 64 kDa protein could be precipitated from BB/Hagedorn and high-incidence BB rat sera, and from rat islet cell and insulinoma cell proteins (36). Additionally, KT1, a monoclonal antibody derived from BB rat spleen cell fusions was found to be cytotoxic to both islet cells and RINm5F cells, and reacted with a 68 kDa protein found on RINm5F cells (37). More recently, 65 and 67 kDa proteins have been associated more directly with diabetes in human, non-obese diabetic (NOD) mice and BB rats. These proteins were identified as two forms of glutamic acid decarboxylase (GAD) that are expressed on β-cells (38) and are perceived as an autoantigen. These two proteins identified by BB rat sera and the monoclonal antibody KT1, may be the different GAD isoforms.

LYMPHOCYTE POPULATIONS AND FUNCTION IN THE BB RAT

LYMPHOPENIA: A severe lymphopenia in the BB rat appears to co-segregate with MHC gene(s), and is present in peripheral blood, lymph node, spleen and thymus affecting T

lymphocytes mostly, but also B lymphocytes (15, 39, 40). Lymphopenia is characterized by a decrease in the overall numbers of lymphocytes, with a reduction of T cell receptor alpha chain and beta chain positive (TcRα/β)$^+$ W3/25$^+$ T_h lymphocytes and absence of TcRα/β^+ OX-8$^+$ $T_{c/s}$ lymphocytes, and increases in natural killer (NK) OX-19$^-$ OX-8$^+$ cells, in double-negative CD5$^-$ cells and double-negative TcRα/β^+ cell numbers (15, 40-45). However, diabetes has also been shown to develop in BB rats having normal numbers of peripheral blood and splenic T_h and $T_{c/s}$ lymphocytes (46).

RT6$^+$ T LYMPHOCYTES AND DIABETES: RT6 is a non-glycosylated cell surface antigen existing as two isomers, RT6.1 of 24-26 and 35 kDa, and RT6.2 of 24-26 kDa proteins transcribed from the RT6^a and RT6^b genes (47-49). RT6 is present on about 70% of peripheral T lymphocytes (45% of T_h and 70% of $T_{c/s}$ lymphocytes), and is absent on bone marrow (BM) cells and thymocytes (47, 48). The absence of RT6 expression on DP rat peripheral W3/25$^+$ and OX-19$^+$ OX-8$^+$ T lymphocyte subsets accounts almost completely for the T lymphopenia (47). However, RT6.1 protein was detected by immunoprecipitation indicating that the RT6^a structural gene is intact (49).

An intrinsic defect is thought to exist in DP rat prothymocyte development because neither DP BM nor prothymocytes generate RT6$^+$ T lymphocytes (50). Normal pluripotent haematopoietic stem cell and TdT$^+$ cell numbers in DP BM and thymocytes suggest, rather, a defect in post-thymic lymphocyte maturation (51). However, normal post-thymic differentiation is thought to occur since DP rat lymphocytes demonstrate normal restriction fragments of RT6^a and RT6^b genes (52), normal transcripts, the presence of a small number of peripheral RT6$^+$ lymphocytes (53), and the presence of RT6$^+$ intraepithelial T lymphocytes (54). The absence of RT6$^+$ T lymphocytes in the BB rat may result from defects in thymic stroma integrity or in accessory factors associated with lymphocyte maturation/differentiation or through extra-thymic apoptosis.

Depletion of RT6$^+$ T lymphocytes in DR rats results in insulitis and diabetes, and Concanavalin A (Con A)-activated spleen cells from acutely diabetic DP or RT6-T lymphocyte-depleted DR rats passively transfer diabetes to young RT6-depleted DR rats (55). Since RT6 expression is associated with mature, differentiated T lymphocytes which may possess suppressor activity, these results suggest that RT6$^+$ T lymphocytes play an important immunoregulatory role in preventing diabetes.

Activation of endothelial cells (EC) appear to be affected by RT6-T lymphocyte depletion and express increased levels of Class I and Class II MHC antigens, similar to EC cultured with interferon-gamma (IFN-γ) (56). Induction and expression of MHC antigen and intercellular adhesion molecule 1 (ICAM-1) on EC required IFN-γ mediated through the contact with T lymphocytes (56). This suggests that RT6-depleted lymphocytes have the capacity to activate EC which may be important in recruiting lymphocytes to the islet. The absence of RT6$^+$ T lymphocytes may be a consequence of lymphopenia not related to diabetes development (57). Depletion of RT6$^+$ T lymphocytes and inoculation with sterile rat faeces significantly increases diabetes, acute peritonitis and mild pancreatitis (58). Mature differentiated RT6$^+$ T lymphocytes may be able to regulate autoreactive T lymphocytes by exerting a suppressor function, thereby preventing diabetes development.

HELPER T LYMPHOCYTE SUBSETS IN THE BB RAT: Rat leukocytes express the heterogeneous common leukocyte antigen (L-CA), CD45, which in the $CD4^+$ T lymphocyte subset exists as 180, 190, 200, and 220 kDa glycoproteins resulting from alternative splicing of CD45 transcripts. Rat T_h lymphocytes can be phenotyped into two CD45 subsets using the monoclonal antibodies, OX-22 or OX-32: $CD4^+$ $OX\text{-}22^+$ (CD45RA/RB) T_{h1} lymphocytes produce interleukin 2 (IL-2), IFN-γ, and lymphotoxin (LT), are involved in graft-versus-host disease, respond to alloantigen in mixed lymphocyte culture (MLC) and to lectin stimulation, but are ineffective in helping primed B lymphocytes to produce antibody and $CD4^+$ $OX\text{-}22^-$ (CD45RO) T_{h2} lymphocytes produce interleukin 4 (IL-4) and interleukin 5 (IL-5) and have the ability to help B lymphocytes produce antibody (59, 60).

BB rat peripheral T lymphocytes are $CD45RA/RB^-$ and $RT6^-$, and the absence of both subsets account for the T lymphopenia (61). This absence could arise from a post-thymic maturational defect in which precursor T_h lymphocytes are unable to differentiate towards either T_{h1} or T_{h2} lineages. Both CD4 and CD8 lymphocyte subsets from DP rat lymph nodes and intestinal intraepithelium are $CD45RA/RB^-$, as are islet infiltrating T lymphocytes (54, 61, 62). These $CD45RA/RB^-$ $RT6^-$ lymphocytes strongly correlate with lymphopenia, defective CD45R expression and aberrant TNF production (63).

There appears to be "holes" in the thymic stroma of DP rats and the thymocyte phenotype in these "holes" are exclusively $CD4^+$ $CD8^+$ (64). As a result, thymocytes in the DP may be autoreactive in nature but not devoid of RT6 expression (64).

There are increased numbers of immature, and reduced numbers of mature, $CD4^-$ $CD8^+$ cells in the DP rat thymus (65). However, these immature thymocytes respond normally to IL-2 + anti-TcR Ab and CD5 lymphocytes co-express TcR$\alpha\beta$ (65). This suggests that defects in peripheral T lymphocytes results from abnormalities beginning at the thymic level. Thymic antigen presenting cells (APC), of macrophage or dendritic cell origin may be responsible for the thymic defect since passive transfer of MHC-matched thymic APC to DP rats results in normal ontogeny and normal T lymphocyte responses (66). No RFLP are associated with BB rat TcR α chain constant region (67); however, there does appear to be limited usage of the TcR V_β chain in peripheral T lymphocytes but not thymocytes (68).

DP rat lymph node T lymphocytes respond equally as well as WF T lymphocytes following stimulation with R73 (TcR mAb), or Con A whereas cross-linking CD4 prior to R73 results in WF but not DP T lymphocyte apoptosis (69). Furthermore, CD4 inhibition of the mixed lymphocyte reaction (MLR) was less effective with DP than WF lymphocytes (69). DP rat lymphocytes successfully rearrange their TcR and are positively selected for self-MHC, but remain as immature T lymphocyte precursors in the periphery due to ineffective CD4 co-receptor signalling (69). These results suggest that T lymphocytes which emigrate from the thymus do not differentiate and mature into $RT6^+$ $CD45R^+$ cells, are associated with the T lymphopenia, and have altered CD4 co-receptor signalling. Together, these phenomena may contribute to the susceptibility of DP rats to develop spontaneous diabetes. However, it was demonstrated that $RT6.1^{+dull}$ cells do exist in DP rats and are generally $CD45RC^-$ with only few $CD45RC^+$ cells found in lymph node T lymphocytes (70).

Differentiated, mature T lymphocytes may be memory cells that participate in regulating the activation and effector functions of autoreactive T lymphocytes by suppressing their clonal expansion and diabetes development. In contrast to DP rats, $RT6^+$-lymphocyte depleted DR rat insulitis is characterized by the presence of $CD45^+$ cells (71).

These results suggest that DP rat T lymphocytes can rearrange their TcR and are positively selected for self-MHC. These lymphocytes do, to a limited extent, express RT6 and CD45RC, but alterations in the thymic stroma, presumably at the level of the thymic APC, results in inefficient CD4 co-receptor signalling, and development of autoreactive lymphocytes which can participate in diabetes development.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ANTIGEN EXPRESSION ON BB RAT LYMPHOCYTES: Activation of the immune response and hence, T lymphocytes results in increased expression of MHC Class II antigens (Ia) on their cell surfaces (63). Both normal and hyperglycaemic BB rats have increased numbers of Ia^+ thoracic duct T_h and $T_{c/s}$ lymphocytes. This increased Ia expression on peripheral blood leukocytes and peripheral blood and lymph node T lymphocytes correlates with those DP rats that would become diabetic (72-75).

Increased Ia cell surface expression correlates with increased *RT1*.D_α and *RT1*.D_β steady state mRNA levels in isolated splenic lymphocytes from prediabetic BB rats only (76). Splenic $CD5^+$ Ia^+ lymphocytes were greater in DP and diabetic DP rats (45), and Ia^+ lymphocytes are found in the islet lesion of $RT6^+$ lymphocyte-depleted DR rats (71). This indicates the presence of an active immune response prior to the onset of overt diabetes and is reflected by increased Ia cell surface protein and mRNA in circulating T lymphocytes. These findings provide additional support for the notion BB rat diabetes is autoimmune and T lymphocyte-dependent in nature.

RESPONSES *IN VITRO* BY BB RAT T LYMPHOCYTES: Unseparated and purified BB rat lymphocytes proliferate poorly in syngeneic and allogeneic primary MLC, to mitogens alone, or after addition of T cell growth factor, IL-2 containing Con A-stimulated supernatants, or purified IL-2 (77-79). In addition, BB rats are unable to reject skin grafts from MHC-compatible and -incompatible rats (79). However, removal of Fc receptor-bearing cells improved mitogen responses and to Con A-stimulated supernatants (79). Unlike lymphocytes, BB rat thymocytes respond normally to mitogens and to Con A-stimulated supernatants (79).

Decreased mitogen responsiveness and variable IL-2 production by BB rat lymphocytes is attributed to deficient $W3/25^+$ lymphocyte numbers (79, 80). The inability of BB rat lymphocytes to respond normally to syngeneic and allogeneic MHC antigens, and to mitogens suggest that a functional defect may exist at the level of the T lymphocyte or may be due to suppression by macrophages.

Depletion of macrophages from BB rat spleen cells, or the addition of indomethacin, increased proliferation and IL-2 production in response to mitogen suggesting that interleukin 1 (IL-1) production was normal (81). Similarly, cell-sorter purified DP rat T lymphocytes respond normally to mitogen although IL-2 production is significantly lower (82).

Adherent DP rat cells markedly suppressed W-line lymphocyte proliferation in response to mitogen and this was also reversed by indomethacin (82). These results suggest that macrophages or macrophage products such as prostaglandins are capable of suppressing responses to antigens and mitogens.

Defective IL-2 receptor (IL-2R) expression on BB rat lymphocytes is suggested by low IL-2 production and the inability to proliferate in response to the addition of IL-2. *In vitro* stimulated DP rat T lymphocytes have significantly lower numbers of IL-2R$^+$ lymphocytes existing as both low and high intensity IL-2R$^+$ populations, compared to DR rat T lymphocytes which exist as a single high intensity IL-2R$^+$ population (82, 83). Northern blot analysis of DP rat lymphocytes reveals few IL-2R p55α and p70β chain transcripts compared to DR lymphocytes (83). However, CD5$^+$ splenic lymphocytes from DP and diabetic BB rats express greater levels of IL-2R compared to DR and WF rats (45). These results suggest that DP rat lymphocytes are difficult to activate and this may be due to macrophage-mediated suppression.

Lymphopenia in BB rats is associated with the absence of OX-19$^+$ OX-8$^+$ T lymphocytes (43), however, significant numbers of these cells are generated after Con A or MLC stimulation, but are unable to lyse allogeneic targets in a cell-mediated lympholysis (CML) assay (44). The addition of MLC-supernatant, or purified IL-2, and IFN-γ also fail to generate any cytotoxic activity in either a CML assay or in a lectin-dependent cytotoxicity assay (44). There are no increases in the number of OX-19$^+$ OX-8$^+$ or W3/25$^+$ cells generated after lymphocytic choriomeningitis virus (LCMV), and no cytolytic activity against LCMV-infected targets, suggesting that DP rats do not have functional OX-19$^+$ OX-8$^+$ lymphocytes (84). This further suggests that a central defect in the thymus or defects in the lymphocyte itself are responsible for the inability of DP rat T lymphocytes to be activated and functional.

DP rats are unable to generate functional $T_{c/s}$ lymphocytes. However, some DP rat OX-8$^+$ OX-19$^-$ cells express TcR suggesting that these cells are CTL (85). Lower levels of OX-8 and absent OX-19 expression on DP rat lymphocytes may correlate with reduced cytolytic activity. Target cells expressing greater amounts of Class I MHC antigen are lysed by DP rat CTL (85). These findings imply that BB rat lymphocytes may not undergo further post-thymic differentiation/maturation (85) and may result in the inability to develop into functional $T_{c/s}$ lymphocytes.

NATURAL KILLER (NK) CELLS IN THE BB RAT: There are increased numbers of peripheral blood and splenic NK cells, and increased NK cell-mediated cytolysis in DP rats (86). In LCMV-infected DP rats, NK cell activity is greater compared to DR rats up to 7 days post-infection (84). Splenic NK cell numbers are two-fold greater in DP and diabetic BB rats compared to DR and WF rats (45) suggesting that increased numbers of NK cells may contribute to diabetes development by killing islet cells in an MHC-unrestricted manner.

ISLET-CELL SPECIFIC T LYMPHOCYTES IN THE BB RAT: Helper T lymphocyte lines and hybridomas isolated from spleen and pancreas of newly diabetic BB rats proliferate in response to islet cell or RINm5F cell antigen in the context of *RT1^u* accessory cells, and produce significant amounts of IL-2, but are not cytolytic to either islet or RINm5F cells (87,

88). Proliferation and IL-2 release by these cell lines and hybridomas can be inhibited by the addition of anti-*RT1*^{u} serum or an anti *RT1*.D monoclonal antibody (89, 90). More recently, DP BB/Wor rat-derived CD4$^+$ CD5$^+$ CD8$^-$ T lymphocyte lines were found to be specific for *Escherichia coli* GAD or islet cell antigen, and in the context of *RT1*^{u} APC, these lines proliferate, secrete IL-2 and IFN-γ (91). Identification of DP rat autoreactive T_h lymphocytes which recognize autoantigen, possibly GAD, on the surface of islet cells may be important in diabetes development.

MACROPHAGES AND DENDRITIC CELLS IN THE BB RAT

Macrophages are thought to play an important role in initiating diabetes in the BB rat. During the early stages of insulitis, OX-41$^+$ macrophages are predominant in the islet, followed later by lymphocytes (92), and may be associated with increased splenic OX-41$^+$ macrophage numbers in DP and diabetic rats (45). During the earliest stage of insulitis, OX-18$^+$ OX-3/6/17$^+$ ED1$^+$ W3/25$^+$ macrophages predominate, followed in later stages of insulitis by OX-8$^+$ and W3/25$^+$ lymphocytes (93). Infiltrates contain both inflammatory ED1$^+$ and resting resident ED2$^+$ macrophages which become activated to express the ED1$^+$ ED2$^+$ W3/25$^+$ OX17$^+$ phenotype (94-96). Furthermore, in RT6-lymphocyte depleted DR rats, both ED1$^+$ and ED2$^+$ macrophages are found in islets with ED1$^+$ macrophages being more numerous (71).

Dendritic cells (DC) are thought to be the first to invade the periphery of the β-cell islets prior to lymphocytes and scavenger macrophages (97). In the early stages of insulitis, DC predominate, and are followed later by increased numbers of ED2$^+$ and ED3$^+$ macrophages (98). Basal stimulatory activity of BB rat DC is comparable to WF DC, and this activity is significantly increased after co-culture with activated macrophages, or in the presence of IL-1 and granulocyte-macrophage colony stimulating factor (GM-CSF) (99).

ED3 is specific for sialylated glycoconjugates and is an adhesion molecule specific for activated lymphocytes (100). Glucocorticoids increase ED3 expression while IFN-γ reduces it (100). ED3$^+$ macrophages are generally restricted to lymphoid organs and sites of tissue damage in the late stages of autoimmune disease suggesting that they may be suppressor macrophages (100). The presence of ED3$^+$ macrophages in the damaged islet may act in a protective manner. It is not known what triggers DC or macrophage migration to, and infiltration within, the islets and their role(s) in islet cell killing. One suggestion is that a venular permeability defect throughout the pancreas allows this migration (101). This helps to explain the overall infiltration into the endocrine as well as exocrine pancreas and exocrine cell lysis. ED3 expression on islet macrophages may indicate a cessation of the effector function of T lymphocytes and an overall change in balance of cytokines from the initial predominance of IL-2 and IFN-γ during the early and mid-stages of islet destruction to elevated levels of glucocorticoids and cessation of the diabetic process.

Macrophage depletion in BB rats by chronic administration of silica, prevents diabetes development and thyroiditis (102, 103). Macrophages from silica-treated animals appear enlarged, and have a higher level of activity (104). These results suggest that depletion of, or altering macrophage function, by silica administration could prevent diabetes.

Adoptive transfer of Con A-activated splenocytes from silica-treated DP rats were unable to transfer disease, however, splenocytes from silica-treated acutely diabetic BB rats did

(105). Silica-treated DP rats have significantly reduced numbers of NK cells, T_h and $T_{c/s}$ lymphocytes, and macrophages, and Con A-activated splenocytes from these rats are less cytotoxic to YAC-1 cells, primary rat islet cells and RINm5F cells (105). Silica treatment prevents islet allograft rejection in recipients except in carrageenan-treated recipients (106).

Ingestion of silica particles by macrophages may be sufficient to activate increased monokine production or depletion of macrophages with silica may impair autoreactive T lymphocyte activation and effector function. The combined reduction in macrophage numbers and increased release of macrophage products could suppress autoreactive T lymphocytes and protect against diabetes development.

IMMUNE-MEDIATED ISLET CELL KILLING

COMPLEMENT-DEPENDENT ANTIBODY-MEDIATED ISLET CELL KILLING: Complement-dependent, antibody-mediated cytotoxicity (C'AMC) of rat islet cells can be demonstrated using antibodies from pre-diabetic and overtly BB rats (107). Peak serum cytotoxicity is observed just prior to, or just after, onset of overt diabetes (108). Complement-dependent islet cell surface antigen-mediated cytotoxicity can also be demonstrated in crosses of outbred BB and Long-Evans rats and always correlates with diabetes onset (109). Autologous, allogeneic and xenogeneic islets are destroyed with a combination of sera and mononuclear cells from 40-60 day old DP rats in an antibody-dependent cell cytotoxicity (ADCC) assay and with increased duration of diabetes, ADCC declines (110). Both C'AMC and ADCC increased significantly in diabetic animals that were transplanted with islets (111).

C'AMC has been demonstrated against lymphocytes with variable results (112, 113). C'AMC of islet cells involves the classical complement pathway, requires Ca^{2+} and alternate pathway factor b inactivation (114). BB rats injected intrasplenically with syngeneic islet cell homogenates have an increased C'AMC and also develop diabetes (115). This suggests that islet cell killing can occur in an MHC-independent, antibody-specific cell- or complement-dependent fashion which may be important in targeting islet cells and initiating diabetes onset.

MACROPHAGE-MEDIATED ISLET CELL KILLING: Macrophages may also be important in the development of diabetes by being directly cytotoxic to islet cells. Activated $ED1^+$ peritoneal macrophages are highly cytotoxic for islet cells and is greatly reduced in the presence of silica (116). Macrophage-mediated islet cell cytotoxicity is enhanced with macrophages from those DP rats which become diabetic and appears to be contact-mediated since islet cell lysis occurs only where there is macrophage-islet contact (117, 118). These results suggest that macrophages are capable of killing islet cells directly or through increased release of inflammatory cytokines. However, macrophages are only cytotoxic in those animals that will eventually become diabetic suggesting that they must be activated to be cytolytic, possibly through the release of inflammatory cytokines.

NATURAL KILLER (NK) CELL-MEDIATED ISLET CELL KILLING: Increased NK cell numbers are found in DP rat peripheral blood and spleens (45, 86). Unstimulated and Con A-activated DP or diabetic rat splenic lymphoid cells reduce insulin content of, and kill, RINm5F and islet cells in an MHC-unrestrictive manner suggesting that β-cell cytotoxicity

may also be NK cell-mediated (119). Purified splenic cells exhibit increased cytotoxicity for both MHC-compatible islets and RINm5F cells suggesting that cytotoxicity is both T lymphocyte- and NK cell-mediated. This was confirmed by reducing NK-sensitive target cell lysis with NK cell-depleted cell preparations (119-122). Only IL-2 could reproduce the Con A effects of diabetic rat splenic cell lysis of islet and YAC-1 cells, further suggesting that NK cells may mediate islet cell killing (122). DP rat splenic lymphoid cells could also kill in an antibody-dependent fashion with variable results (123, 124). These findings suggest that NK cells kill in an MHC-unrestricted manner and do not require the presence of islet cell surface antibody to mediate this function.

Supernatants and purified cytolytic granules derived from a rat NK tumour line, RNK, are cytotoxic to YAC-1 cells and WF islets (125). Cytolysin (perforin) and/or NK cell factor NKCF (NK cytotoxic factor) are thought to be the lytic components found in the granules and supernatants.

Anti-NK cell antibody, 3.2.3- or OX-8-treated young DP rats decreased YAC-1 cell lysis *in vitro* by splenic cells, but only from 3.2.3-treated rats without affecting the incidence of, or timing of diabetes development. OX-8 treatment significantly reduced NK cells and OX-8$^+$ T lymphocytes, and reduced the incidence of diabetes suggesting that OX-8$^+$ T lymphocytes and not NK cells are essential in diabetes development (126). These results confirm an essential role for T lymphocytes in the diabetes. Diabetes developed in DR rats treated simultaneously with anti-RT6 and AGM1 (NK-specific) antibodies, again suggesting that NK cells are not essential for islet cell killing (127).

Serine protease-positive OX-8$^+$ lymphocytes are markedly increased in the peripheral blood of DP and diabetic BB rats and these lymphocytes are thought to be NK cells (128). Cytolysin mRNA is detected in inflamed DP islets and in islets of pre-diabetic and RT6 lymphocyte-depleted diabetic DR rats (129). This suggests that NK cells are predominant in DP rat islets while $T_{c/s}$ lymphocytes are predominant in DR islets since infiltrating cells are OX-8$^+$ and 3.2.3$^-$ (129). Overall, these findings suggest that NK cells and OX-8$^+$ lymphocytes can kill islet cells. However, OX-8$^+$ lymphocytes are necessary for DP and DR diabetes development while NK cells may act in a secondary fashion.

PASSIVE TRANSFER OF DIABETES

Lymphocytes, NK cells and macrophages are implicated in the pathogenesis of diabetes. However, the identity of the cell type(s) which initiate diabetes remain elusive. Passive transfer of diabetic BB rat blood or spleen leukocytes to RNC nude (*nu/nu*) mice resulted in insulitis but not diabetes (130, 131). Passive transfer of diabetic BB rat spleen-, lymph node-, BM-, or thymus-derived immune cells to the SWR/JM (*nu/nu*) mouse model does not result in either insulitis or diabetes (132).

Con A-stimulated diabetic BB rat spleen and lymph node cells passively transferred diabetes to DP rats, to cyclophosphamide-immunosuppressed WF rats and (*RT1^u* X BB)F_1 and (*RT1$^{non\ u}$* X BB)F_1 rats, and to W-line rats (133-135). Both Con A-stimulated supernatants and supernatants free from Con A which were derived from acutely diabetic, and from other rats also passively transferred disease (136). A molecule(s) of greater than 50 kDa was identified in this supernatant (136). Lymphocytes from DP or diabetic rats appear to target the islet directly since passive transfer of radio-labelled Con A-activated

spleen cells to susceptible neonates homed to the islet within 5 days, produced focal infiltration within 8 days and diabetes with a mean age of 12.5 days (137). These results indicate that activated diabetic BB rat lymphocytes or their soluble products can passively transfer disease to susceptible recipients and to recipients made susceptible by immunosuppression. Furthermore, these results suggest that BB rat lymphocytes are islet cell antigen-specific and can home to the these cells.

Transfusion of Con A-activated RT6-depleted lymphocytes from YOS rats to 30 day old DR rats, or nude rats induced diabetes before 60 days of age. RT6-depleted mitogen activated spleen cells from PVG ($RT1^c$) rats induced diabetes or insulitis in PVG nude rats (138). Unactivated or activated cells from acutely diabetic DP rats or from DR rats passively transferred insulitis and diabetes to WAG nude rats 14-56 days after transfer without increasing the number of $CD5^+$ lymphocytes (138). Spleen cells and RT6-depleted spleen cells can passively transfer diabetes, however, the identity of the lymphocyte subset necessary to induce diabetes remains unknown.

Phorbol myristate + ionomycin + IL-2 stimulated splenic cells or Con A-stimulated young or diabetic DP rat splenic cells were both capable of passively transferring diabetes to DP rats (139). Phorbol myristate + Con A + IL-2-stimulated splenic cells of which 78.2% were $OX\text{-}19^+$ passively transferred diabetes to > 90% of the recipients, with no diabetes development arising in those rats passively transferred with PMA + ionomycin + IL-2-stimulated surface immunoglobulin $(sIg)^+$ spleen cells (139). Purified TcR^+ $CD4^+$ lymphocytes stimulated with PMA + ionomycin + IL-2 passively transferred diabetes to recipient DP rats (139). Activated TcR^+ $CD4^+$ T lymphocytes appear to be able to initiate diabetes development.

3.2.3-depleted and non-depleted DP BB rats passively transferred with PMA + ionomycin + IL-2-activated $CD8^+$ T-lymphocyte enriched splenocytes developed diabetes suggesting that NK cells are not required for diabetes development (140). Unseparated T-lymphocytes also passively transferred diabetes to untreated or 3.2.3-depleted recipients suggesting that $CD8^+$ lymphocytes but not NK cells are necessary for passive transfer of diabetes (140). These results suggest that activated whole or RT6-depleted splenic lymphocytes, activated purified T lymphocytes, and more importantly activated T lymphocytes of both the CD4 and CD8 subsets are necessary to passively transfer diabetes.

CYTOKINES AND ISLET CELL KILLING

Pancreatic β-cells are susceptible to various mechanisms of immune cell-mediated killing, and include those soluble products released from immune cells such as macrophages and T lymphocytes. Peripheral blood mononuclear cell-derived supernatants reduced the amount of insulin and glucagon released by islets, and lysed the islets (141) suggesting that lymphokines or monokines can suppress insulin release and be directly cytotoxic to islet cells. However, the identity of the factors found in these supernatants remain to be identified.

INTERLEUKIN 1 (IL-1): IL-1 inhibits islet cell insulin secretion, and insulin and glucagon content, and killed islets (143). Islet cell sensitivity to IL-1 occurs independently of age, sex, and genetic background, but does appear to depend on their metabolic state (144). *In vitro* culture of islet cells in high serum or high glucose reduced the toxicity of IL-1,

although the effect by sera may have been indirect and due to the presence of IL-1 inhibitors (144). Whereas low amounts of IL-1 stimulates increased insulin release, and increased pro-insulin biosynthesis in a time-dependent manner, high concentrations selectively decreased pro-insulin biosynthesis and prepro-insulin mRNA indicating that IL-1 acts at a pretranslational level of insulin biosynthesis (145, 146).

TUMOUR NECROSIS FACTOR ALPHA/BETA (TNF-α/β): Initially, TNF-α/β was shown not to be toxic to islet cells (142, 145). However, high concentrations of TNF-α alone, or low concentrations of TNF-α together with IL-1 inhibited islet cell insulin content and release (147). Furthermore, the combination of IL-1, TNF, and IFN-γ, can inhibit insulin release and be directly cytotoxic to rat islet cells maintained in monolayer culture (148-150). Lymphotoxin (LT), also called TNF-β, alone does not alter islet morphology and does not enhance the effect of IL-1β and the combination of IFN-γ and LT does not affect insulin release or islet morphology (150). However, rat or mouse IFN-γ + IL-1β, with TNF, or with LT is cytotoxic to rat islet cells (151). Long-term pre-culture of islet cells with IFN-γ sensitizes them to the cytotoxic effect of TNF or IL-1 (152). There is an inverse correlation between the expression of MHC Class II antigens on islets and cytotoxicity whereby peak expression of Class II antigens precedes IFN-γ + TNF-α-mediated cytotoxicity (153).

TNF-α alone, or in combination with IFN-γ is also cytotoxic to glucagon-producing alpha (α)-cells (153). IL-1β reduced non-insulin DNA synthesis by 50% and glucagon content by 95% in islets (154). Therefore, macrophage derived cytokines such as IL-1 and TNF-α can act in synergy with T lymphocyte-derived cytokines such as IFN-γ and LT to inhibit insulin biosynthesis and to be directly cytotoxic to the pancreatic islet cell.

INTERLEUKIN 6 (IL-6): IL-6 is another macrophage product that inhibits glucose-stimulated insulin secretion without altering insulin content and induces islet cell apoptosis (155). Moreover, IL-1-treated islet cells produced IL-6 (155). Culture supernatants from murine islets contain low but detectable levels of IL-6 which are increased 6-fold in the presence of IFN-γ, 40-fold in the presence of TNF-α and 115-fold in the presence of IFN-γ + TNF-α which peaks at 48 hours of culture. Peak IL-6 mRNA levels are present at 24 hours of culture and is similar for RINm5F cells (156). While IL-6 alone can inhibit glucose-stimulated insulin secretion, the combination of IL-1, TNF and IL-6 contributes to islet cell cytotoxicity (157). These findings suggest that exogenous as well as endogenous cytokine production can lead to islet cell death.

The functional state of the islet cell itself may determine its susceptibility to cytokine-mediated killing. Greater cytotoxicity of islet cells to IL-1, TNF-α or IL-1 + TNF-α occurs in the presence of 11 mM glucose than in the presence of lower concentrations. This suggests that as islet cells are killed, the remaining β-cells increase their activity and may also become more susceptible to killing (158). Interleukin 1-, TNF-α- and IL-6-producing cells were found within the islets and in the exocrine pancreas of diabetic BB rats with TNF-α-positive cells being the most predominant (159). These results suggest that in addition to the lethal effects of lymphocytes, NK cells and macrophages on β cells, soluble products from these immune cells including IL-1α/β, TNF-α, LT and IL-6 can mediate β cell injury and destruction.

Differentiated endocrine cells such as β-cells are sensitive to (pro)-inflammatory cytokines and can also produce some of these cytokines after stimulation. A mouse β-cell line, β-TC1, derived from mice carrying a hybrid insulin-SV40 tumor antigen transgene, expresses TNF-α mRNA and protein after culture with low doses of IL-1β (160, 161). These results suggest that islet cell damage and death may occur by IL-1-induced TNF-α-mediated killing.

TRANSFORMING GROWTH FACTOR BETA (TGF-β): Some cytokines may be protective rather than cytotoxic for islet cells. This may be important in inhibiting or reversing inflammatory cytokine-mediated islet cell damage. Islet cells treated with TGF-β for up to 2 weeks prior to, or concurrently in an *in vitro* lymphocyte-mediated killing assay or cytokine (TNF + IFN-γ)-mediated killing assay, significantly reduced islet cell killing (162, 163). This suggests that TGF-β can either inhibit or reverse the cytotoxic potential of TNF + IFN-γ and prevent islet cell damage. Furthermore, TGF-β may also act directly on the islet cell making it more resilient to attack by immune cells or their products. Transforming growth factor β1, TGF-β2 and TGF-β3 mRNA and protein are detected in normal adult pancreatic islets and may play a role in preventing immune killing. This suggests that in islets from diabetes-susceptible individuals, a disturbance in TGF-β production may exist rendering the islet to enhanced susceptibility to immune-mediated damage (164).

INTERLEUKIN 10: IL-10 is an anti-inflammatory cytokine produced by T_{h2} lymphocytes, B lymphocytes and macrophages and may be important in the treatment of autoimmune diseases (165). IL-10 increases islet cell insulin accumulation by about 50% and islet insulin content by 30-50% (166). However, pre-treatment of islets with IL-10 fails to protect against, or reverse, the damaging effects of IL-1β (166).

Directed IL-10 expression in islets from transgenic mice transplanted as allografts in MHC-incompatible hosts were still susceptible to mononuclear cell infiltration and rejection although significant amounts of IL-10 could be detected by immunohistochemistry (167). Double transgenics in which islets expressed IL-10 and LCMV glycoprotein (GP) or nucleoprotein (NP) had insulitis and diabetes (167). These findings suggest that IL-10 is unable to inhibit an active cell-mediated response against the GP- or NP-expressing islets and once initiated, a cellular immune response is unaffected by the actions of IL-10. Interleukin 10 is unable to suppress inflammatory cytokine action on, or the effector function(s) of inflammatory cells infiltrating the islet.

Interleukin 1 stimulates islet cell production of another anti-inflammatory agent, prostaglandin E_2 (PGE_2) (168, 169). The addition of cyclooxygenase inhibitors to islet cell cultures suppressed islet cell PGE_2 production, but did not prevent IL-1 inhibition of glucose-induced insulin secretion (168, 169). A natural inhibitor of IL-1 extracted from urine inhibited IL-1-stimulated islet PGE_2 production and inhibited the decrease in insulin content (169). Taken together, protective mechanisms may exist, in or nearby islets ensuring that the actions of inflammatory cytokines such as IL-1, TNF, and IFN-γ are not detrimental to the insulin-producing cells. However, if imbalances in protective and destructive cytokines exist, cytokines may inhibit insulin secretion and kill islet cells. Finally, these findings demonstrate that an additional mechanism of islet cell killing is cytokine-mediated leading to overt diabetes.

MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PANCREATIC ISLET β-CELLS

Cell surface expression of MHC antigens on cells of immune and non-immune origin can be regulated by IFN-γ (170), and/or TNF-α (171), and inappropriate MHC antigen cell surface expression may contribute to autoimmune disease (172).

Class I and II MHC cell surface antigen expression was demonstrated on human pancreatic β-cells of newly manifested IDDM (173), suggesting that aberrant expression of MHC molecules on β-cells may be important in diabetes. Only low levels of Class I MHC antigens were detected on freshly isolated or 2 day-cultured mouse pancreatic islets and increased 2-10 fold on 50-98% of the islets after culture with IFN-γ (174). Culture of islets for more than 6 days with IFN-γ or a lymphokine supernatant increased Class II MHC antigen expression (175). At least 50% of β-cells from high incidence BB but not low incidence BB or WF were induced to express Class II MHC antigens after 5 days of culture with IFN-γ (176).

Low levels of Class I MHC antigens (HLA-A, B, C), but not HLA-DR could be detected on adult human β cells and these levels increase 2-6 fold on adult and 17-20 fold on fetal β-cells after exposure to IFN-γ (177). However, insulin-containing islet cells in pancreatic sections from diabetics did stain for HLA-DR (178). The addition of TNF-α to IFN-γ increased greater expression of HLA-DR on human α and β cells (179). Furthermore, IFN-γ, together with TNF or LT, but not IL-1α/β increased Class II MHC expression on β cells (180, 181). While results are variable for MHC Class II expression, it is possible that inflammatory cytokines, if present in sufficient amounts, can enhance Class I and induce Class II MHC antigen expression on β cells.

RINm5F cells cultured with 10% Con A stimulated lymphocyte supernatants or with low amounts of IFN-γ had increased Class I MHC expression, whereas moderate amounts of IFN-γ induced Class II MHC expression (182, 183). Isolated LEWIS rat islets cultured with rat IFN-γ for up to 15 days were negative for Class II MHC antigen expression, however, islets cultured for 7 days followed by culture for 6 days with IFN-γ were positive for both Class I and Class II MHC antigens (184). Pre-diabetic BB and BB-derived rat islets, and RINm5F cells cultured with 5% Con A stimulated lymphocyte supernatants had increased levels of Class I MHC RNA (185, 186). While consistent increases in Class I MHC expression are observed in islets and RINm5F cells, Class II expression is variable and may depend on the sequence of, and exposure time to cytokines.

Islet preparations express class I MHC heavy chain RNA which increased with time in DP and WF rats (187). Peak Class I MHC heavy chain RNA was observed in islets from 50 day old DP rats and from newly diabetic BBUF rats and were 3-fold and 2-fold greater, respectively, compared to those levels in WF islets (187). No 30 d DP islets contained detectable levels of Class II α-chain RNA. However, in 50 d BB and newly diabetic BBUF, Class II expression was observed and expression was limited to islet dendritic cells (187).

RINm5F cells cultured with crude lymphokine preparations or IFN-γ only expressed enhanced Class I MHC heavy chain RNA and protein (188, 189). Insulin-containing human islets from recent onset diabetes expressed Class II MHC antigens and over-expressed of Class I MHC antigens (190). When RINm5F cells were cultured in the presence of IFN-

γ, generalized Class I and focal Class II MHC expression was enhanced and increased in proportion to the dose and duration of IFN-γ exposure (191). Interferon-γ or TNF-α increased Class I but not Class II MHC antigen expression on normal human and mouse β-cells, and RINm5F cells after culture for 5 or 6 days, while IFN-γ + TNF-α increased both Class I and Class II MHC antigen expression (192).

These findings suggest that release of cytokines such as IFN-γ, TNF-α and possibly LT and IL-1α/β by macrophages and lymphocytes in the vicinity of the β cell can induce aberrant MHC antigen expression possibly rendering these cells more susceptible to killing.

PREVENTION OF DIABETES IN THE BB RAT: IMMUNOSUPPRESSION

BONE MARROW TRANSPLANTATION: Insulin-dependent diabetes mellitus is an autoimmune disease involving the T lymphocyte as the effector cell indicating that susceptibility to disease may reside in lymphocyte stem cells in the BM. Neonatal BB rats injected with BM from WF or (ACI X BB)F_1 prevents diabetes development and improves immune cell function by normalizing lymphocyte numbers and increasing the primary MLR (193-197). However, diabetes was not completely prevented in rats receiving neonatal transplantation of T lymphocyte-depleted WF BM. These recipients were profoundly lymphopenic similar to untreated DP rats, and cells from these rats responded weakly in an allo-MLR (198, 199). DP rats reconstituted with DR BM are insulitis- and diabetes-free, whereas DR rats reconstituted with DP BM developed accelerated insulitis and diabetes compared to DP rats (200). Wistar Furth, BN/WF, BN/Yos, DP, and DR rats all of which are $RT1^u$, when reconstituted with DP BM and passively transferred with acutely diabetic DP rat Con A-activated spleen cells, developed diabetes and insulitis (200). DR rats had a 100% incidence suggesting that a defect exists in DP rat BM (200).

These results indicate that a probable genetic defect in the BM participates in the diabetes process and can be corrected with normal syngeneic BM transplantation. Apparently, the BM cell that may be responsible for this protection is the "contaminating" T lymphocyte since T lymphocyte-depleted BM did not protect against disease development. This does exclude a possible thymic defect in diabetes development. Both a defect in BM and in the thymus may be responsible for diabetes development.

NEONATAL THYMECTOMY AND THYMIC TRANSPLANTATION: Partial or complete neonatal thymectomy of DP rats reduces the incidence of diabetes to 9 and 3% respectively and increases lymphopenia by further reducing $W3/25^+$ lymphocyte numbers suggesting that diabetes in the BB rat is a thymus-dependent cell-mediated event (201). Lethally irradiated, thymectomized DP rats injected with DP BM and transplanted with a nondiabetic BB rat thymus or lethally irradiated, thymectomized non-diabetic BB rats injected with non-diabetic BB BM and transplanted with a DP rat thymus had no change in the numbers of lymph node or thoracic duct T and B lymphocytes and had an unaltered primary allo-MLC response (202). However, DP BM chimeras passively transferred with non-diabetic BM had elevated T lymphocyte numbers whereas irradiated non diabetic BB rats passively transferred with DP BM had reduced T lymphocyte numbers suggesting the presence of a BM defect (202).

Irradiated BB rats passively transferred with T lymphocyte-depleted WF BM had a significant decrease or delay in diabetes development, were less lymphopenic with incomplete W3/25 and OX-8 subset restoration with a normal response to alloantigen (203). In contrast, WF rats passively transferred with BB rat BM did not develop diabetes, but did develop a characteristic lymphopenia with a normal response to alloantigen (203).

BB rats transplanted with γ-irradiated neonatal (DA X BB)F_1 thymus were protected against insulitis and diabetes development, and had normal T lymphocyte responses whereas BB rats transplanted with dGua-treated thymus developed a severe insulitis and diabetes and poor T lymphocyte responses similar to untreated DP rats (204-206). Bone marrow transplantation followed by neonatal thymus transplantation fails to reduce the incidence of diabetes in BB rats or improve the lymphopenia (206).

LYMPHOCYTE TRANSFUSION: Weekly transfusions of whole blood prevents diabetes and insulitis, and increases peripheral lymphocyte responses to Con A stimulation (207). White cell-transfused animals are prevented from developing diabetes and insulitis and had significant increases in lymphocyte subset numbers (208). However, transfusion of DP rat spleen cells slightly increases diabetes (208). T lymphocyte-depleted blood transfusion slightly decreases diabetes and both whole blood and T lymphocyte-depleted blood recipients respond better to Con A stimulation (208). Protection against diabetes and insulitis in BB rats is MHC-restricted and observed only with syngeneic spleen cells (209).

While lymphocytes can protect against diabetes development, the identity of the cell type which confers this protection remains unknown. Transfusion of W3/25-enriched T lymphocytes but not OX-8-enriched or OX-8 + W3/25-enriched T lymphocytes protected against diabetes and insulitis and significantly increased proliferative responses to Con A (210). However, *in vivo* depletion of W3/25 or OX-8 lymphocytes with antibodies and passive transfer of lymphocytes from these donors protected recipients against diabetes (210).

Neonatal DP rats receiving intraperitoneal or intravenous injections of splenocytes or Con A blasts from syngeneic rats were protected against diabetes in a dose- and time-dependent manner, but were not protected against passively transferred diabetes with diabetic BB rat Con A blasts (211, 212). Furthermore, lymphocytes derived from normoglycaemic DP rats could passively transfer protection against diabetes but could not protect against destruction of islet grafts (213). This indicates that passive transfer of intact functional thymuses from normal rats, and equally important, mature lymphocytes can infer protection against diabetes development. Lymphocytes from normoglycaemic DP rats may have matured sufficiently to act as immunoregulatory cells preventing diabetes.

ANTIBODY-MEDIATED PREVENTION: Autoreactive T lymphocytes are responsible for diabetes development and suppression of the effector activity of these cells may infer protection against disease. Chronic treatment with an anti-lymphocyte serum (ALS) (214), or an anti-I-E but not anti-I-A Class II MHC mAb (215), or OX-19 (pan-T lymphocyte), or OX-8 ($T_{c/s}$ lymphocyte and NK cells) and OX-17 (Class II MHC I-E) significantly prevents against diabetes development in DP-BB/Wor rats, whereas treatment with antibodies to W3/25$^+$ T_h lymphocytes does not (216). Reappearance of diabetes after islet

transplantation is also prevented in OX-8 but not W3/25 antibody-treated animals (217). Targeting of specific lymphocyte subsets is capable of preventing diabetes.

Therapy with anti-AGM1, which recognizes NK cells among other cells, prevents destruction of WF islets in WF-tolerant BB rats by reducing the number of OX-8$^+$ lymphocytes and YAC-1 cell-specific lysis (218). However, chronic treatment with 3.2.3, a rat NK-specific antibody, is not protective compared to OX-8 (126). This suggests that diabetes development is T lymphocyte-mediated and not NK cell-mediated, and indicates that DP rats do have OX-8$^+$ T lymphocytes.

Chronic adminstration of an anti-CD2 mAb which recognizes T lymphocytes, thymocytes, NK cells, and splenic macrophages protects against insulitis and diabetes development in BB rats, reduces adoptive transfer of disease to DP rats, to RT6$^+$-cell depleted DR rats, and poly [I:C]-treated DP rats but not RT6$^+$-cell depleted, poly [I:C]-treated DR rats (219). These findings indicate that T lymphocytes are the effector cells in diabetes.

Antibodies to the IL-2R, alone or in combination with cyclosporin A (CsA), effectively prevents rejection in LEW.1W MAxK (RT1^u) rats which received islet grafts under the kidney capsule (220). Newly hyperglycaemic BB rats receiving CsA together with an anti-rat IL-2R antibody regain about 50% of their pancreatic insulin and demonstrated a marked reduction in IL-2R$^+$ and Class II MHC antigen-expressing lymphocytes (221). These findings suggest that suppression of activated lymphocytes and inhibition of IL-2 action is sufficient for protection against diabetes.

PHARMACOLOGICAL PREVENTION OF DIABETES: Cyclosporin A (CsA) inhibits transcription of cytokine genes such IL-1 and TNF-α in the macrophage, IL-2 and IFN-γ in lymphocytes, and may generate suppressor macrophages (222-225). Cyclosporin A may suppress either T_h lymphocytes or $T_{c/s}$ lymphocytes and induce suppressor macrophages which could participate in diabetes prevention.

Cyclosporin A-treated DP rats do not develop insulitis or diabetes (226). Newly diabetic BB rats treated with methylprednisolone and anti-lymphocyte serum (ALS) are cured from diabetes and if treatment is initiated prior to overt diabetes development, both the methylprednisolone + ALS and CsA + ALS protect (227).

Prevention of BB rat diabetes by CsA treatment is time-dependent and most effective when initiated between 60 and 70 days of age (228, 229). Both oral and peritoneal routes of CsA administration are equally effective (229). Long term CsA therapy from 6-21 weeks of age protects completely against diabetes, whereas initiation of therapy later or termination earlier provides only partial protection (230). This suggests that CsA therapy must commence at, or near the time in which lymphocytes and possibly macrophages become activated and must continue throughout the "effector" phase to be successful in preventing diabetes.

Cyclosporin A protects against diabetes development, however, the effects of CsA on the immune system remained to be determined. Chronic or intermittent CsA treatment significantly reduces peripheral blood and splenic T lymphocyte (OX-19$^+$) and T_h lymphocyte (W3/25$^+$) numbers (231, 232). These results further suggest that CsA

suppression of autoreactive cells must occur at a time when then immune response first starts and must be continued throughout the "effector" phase.

Concanavalin A-activated lymphocytes from CsA-treated nondiabetic DP rats are significantly less cytotoxic to RINm5F cells when compared to untreated DP rats (233). Furthermore, CsA-treated DP rats have decreased numbers of T lymphocytes (OX-19$^+$) and W3/25$^+$ T_h lymphocytes, reduced IL-2 production, but significantly increased numbers of NK cells (233). Immunohistochemical analyses of pancreases from CsA-treated BB rats are free from Class I MHC antigen hyperexpression, islet infiltration and markedly decreased extraislet ED1$^+$ macrophages (234). This indicates that CsA treatment does not only affect lymphocyte activity but also inhibits lymphoid cell cytokine production which could act on islet cells rendering them more susceptible to lysis.

Thalidomide can prevent graft versus host disease after BM or renal transplantation and has a synergistic effect when used in combination with CsA (235). Thalidomide decreases the CD4:CD8 ratio by reducing CD4 and increasing CD8 lymphocyte numbers (236), decreasing NK cell numbers, increasing the number of mature B lymphocytes, and decreasing cell adhesion and integrin molecules on lymphocytes and monocytes (237). Thalidomide-treated BB rats were not protected against diabetes or lymphocytic thyroiditis development (238). The lack of protection may be due to the ability of thalidomide to selectively inhibit TNF-α production by monocytes (239) and enhance TNF-α mRNA degradation (240). Decreasing or inhibiting TNF-α in BB rats does not protect against diabetes development suggesting that this cytokine may be important in protecting against diabetes development.

VIRUSES: Viruses implicated in diabetes development include Coxsackievirus B4 (241), congenital rubella (242), retroviruses (243), reovirus types 1 and 2 (244, 245), encephalomyocarditis virus D variant and mengovirus (245), mumps virus (245), cytomegalovirus (245), which can kill islet β cells directly, and Kilham's rat virus which kills lymphocytes (246).

Chronic lymphocytic choriomeningitis virus (LCMV) clone 13 infection of DP rats prevents diabetes and was associated with a further transient reduction W3/13$^+$, W3/25$^+$, and OX-8$^+$ lymphocyte numbers (247, 248). Furthermore, chronic LCMV infection of NOD mice prevented diabetes, and transfer of LCMV-infected lymphocytes to diabetes-prone NOD mice also protected against disease (249). While LCMV may lyse lymphocytes necessary to precipitate diabetes in BB rats and NOD mice, LCMV infection is associated with dramatic increases in splenic cell TNF-α, IL-1α, and IL-1β mRNA levels, and to a lesser extent, IL-6 and IFN-γ mRNA levels, peaking at 3 days post-infection and maintained for over 6 days (250). While viruses may mediate β cell-specific damage or participate in diabetes development, LCMV infection increases the production of pro-inflammatory cytokines such as IL-1α/β, IL-6, and TNF-α. In the NOD mouse and BB rat, these cytokines, or at least TNF-α may play a protective role and prevent diabetes development.

OTHER METHODS: Other methods have been employed to prevent diabetes in the BB rat. Early treatment of BB rats with insulin or diazoxide (an inhibitor or endogenous insulin secretion) prevents diabetes with normal-appearing pancreases and no apparent differences in the lymphocyte subsets (251, 252). Insulin-mediated protection against

diabetes was confirmed in RT6$^+$ lymphocyte-depleted DR/Wor rats, however, lymphocytes from these animals passively transferred diabetes to naive recipients (253). Furthermore, passive transfer of diabetes to insulin-treated BB rats was also prevented (254). This suggests that active insulin-producing β-cells may provide signals necessary for their destruction.

Intrathymic inoculation of DR islet cells into 25 day old DP recipients protects against diabetes, reduces spleen cell-mediated islet cell killing, but not YAC-1 cell killing and does not alter OX-19$^+$ and OX-8$^+$ OX-19$^-$ cells, but increases OX-8$^+$ OX-19$^+$ cells (255). Intrathymic inoculation of WF islets into neonatal DP rats prevented diabetes up to 210 days of age without altering CD4 or CD8 lymphocyte, B lymphocyte, or TCRαβ$^+$ lymphocyte numbers (256). These results indicate that direct exposure to islet cell antigen at the site of self:non-self discrimination, the thymus, possibly directs apoptosis of autoreactive islet cell-specific lymphocytes and is sufficient for the prevention of diabetes.

Total lymphoid irradiation reduces insulitis and prevents diabetes in BB rats and slightly reduces Con A responsiveness without altering peripheral blood or spleen T lymphocyte subsets (257). Whole body γ-irradiation had no effect in preventing diabetes except in DR rats which had an increased incidence of disease (258). This suggests that removal of activated, but not resting lymphocytes by irradiation prevents diabetes.

PREVENTION OF DIABETES: IMMUNOREGULATION

Con A is a T lymphocyte and NK cell-specific mitogen capable of activating T lymphocyte helper and effector function, and in high concentrations, suppressor function. In addition, Con A activates NK cells through the production of IL-2. PHA can also activate T lymphocytes and PWM can activate both T and B lymphocytes. Mitogens may be good candidates for diabetes prevention.

Adjuvants are considered to be biological response modifiers capable of non-specifically activating the immune system and augmenting cytokine production and can be grouped into nonbacterial or bacterial types (259, 260). Adjuvants consisting of bacteria or bacterial products include: complete Freund's adjuvant (CFA) which is composed of an emulsion of killed mycobacteria in mineral oil and is generally used in combination with an antigen to stimulate an immune response to that antigen; *Bacillus* Calmette-Guérin (BCG), bacterial lipopolysaccharide (LPS); *Corynebacterium parvum* (BCG); and a streptococcal cell wall preparation, OK-432 are all capable of activating macrophages (260-262). Because these agents activate macrophages and induce cytokine production, they may be candidates to prevent diabetes.

MITOGENS AND DIABETES: Diabetes occurs spontaneously in the BB rat and in the NOD mouse models of human diabetes mellitus. Intraperitoneal treatment young NOD mice with Con A completely inhibits diabetes without depressing insulitis, but does depress proliferative responses to Con A *in vitro*, and increases blast cell numbers, the CD4/CD8 ratio and CD4$^-$ CD8$^-$ cell numbers (263). Intravenous wheat flour lipopolysaccharide (LPSw) treatment of NOD mice also prevented diabetes whereas intradermal treatment only delayed diabetes development by 9 weeks and resulted in an 80% survival rate, similar to TNF-α treatment of NOD mice (264).

ADJUVANTS AND DIABETES: Biological response modifiers such as adjuvants act by modulating the immune response which may be beneficial in preventing autoimmune disease. *Bacillus* Calmette-Guérin (BCG)-treated NOD mice are protected against diabetes development (265). Spleen cells from these treated animals are unresponsive to alloantigen and mitogen stimulation for up to 19 weeks post-treatment and could be reversed by splenic macrophage removal (265, 266). Insulitis and diabetes can be prevented in recipient mice by passive transfer of BCG-treated mice whole spleen cells or macrophages but not T lymphocytes (265, 267). Cytotoxic T lymphocyte generation is inhibited, NK cell activity is absent, and IL-2 production by lymphocytes is significantly reduced and is associated with a significant decrease in IL-2R$^+$ cell numbers (266). Enhanced Mac-2 expression on significantly increased Mac-1$^+$ macrophages numbers during the first 4 weeks after vaccination suggests induction of differentiation/maturation and suppressor macrophage generation (266). These macrophages may curtail diabetes development by directly suppressing autoreactive effector T lymphocytes as demonstrated by both the decreased number of IL-2R$^+$ cells and reduction in IL-2 production.

OK-432 is a streptococcal preparation derived from a low virulent Su strain of group A *Streptococcus pyogenes*. Weekly intraperitoneal treatment of NOD mice with OK-432 from 4-24 weeks of age prevented diabetes and insulitis, increased mononuclear spleen cell numbers and NK cell activity (261). Weekly intraperitoneal treatment of BB rats with OK-432 from 5-30 weeks prevented diabetes and insulitis and significantly reduced spleen cell-mediated RINm5F cell killing without changing OX-19, W3/25 and OX-8 splenic lymphocyte numbers (262). OK-432 provides the best protection against NOD mouse diabetes development when started from 4 weeks through to 15 weeks (268). However, diabetes was not prevented in OK-432-treated irradiated NOD mice passively transferred with diabetic mouse spleen cell (232). Administration of cyclophosphamide (Cy) was unable to promote diabetes in OK-432-treated NOD mice (232).

Spleen cell number increases in OK-432-treated NOD mice is associated with decreases in Thy1.2$^+$, L3T4$^+$, Lyt-2$^+$ cells and increases in Mac-1$^+$, sIg$^+$, and unstained cells. Spleen cells from OK-432-treated NOD mice had stronger responses to Con A and LPS stimulation (268, 269). Furthermore, OK-432 treatment significantly increased NOD mouse serum TNF, but not IL-1, IL-2 or IFN-γ levels (269, 270). Serum from OK-432-treated NOD or Balb/c mice also prevents diabetes and insulitis in naive NOD mice in a manner similar to recombinant TNF and this could be blocked by simultaneous treatment with anti-TNF antibody (269). These results suggest that OK-432 increases macrophage numbers and endogenous TNF-α production, and this combination may prevent diabetes.

BB rats treated with insulin in CFA delayed diabetes development by about 40 days and tuberculin in CFA reduced the incidence of diabetes by about 70% (271). Footpad injection of 4-10 week old prediabetic NOD mice with CFA prevented hyperglycaemia, and lymphocytes, BM cells or nylon-wool nonadherent radioresistant Thy-1$^-$ cells from these mice alone, or mixed with untreated NOD mouse responder cells reduced proliferation in primary allo-MLR and to mitogen stimulation (272). This suggests that CFA may induce the generation of natural suppressor cells (272). BB rats are also protected against diabetes and insulitis development after peritoneal injection of CFA (273), possibly through the generation of non-specific suppressor cells (274), similar to NOD mice. One

mechanism by which CFA protects against diabetes may be to suppress effector cell function by generating natural suppressor cells.

Pancreatic islet homogenates emulsified in CFA or CFA alone injected into the footpads and intraperitoneally in NOD mice prevented diabetes (275). Whole or Mac-1$^+$-enriched splenocytes from CFA-treated NOD mice mixed with diabetic NOD mouse splenocytes prevented passive transfer of diabetes (275). The protective cells appear to be large Mac-1$^+$ cells which increase in number after CFA treatment (275). Morphologically these cells are large, granular, myeloid precursor cells which had an increased proliferative index modestly increased levels of esterase staining, phagocytosis, IL-1 and TNF-α secretion, and no difference in PGE_2 secretion (275). Spleen cells and to a lesser degree, lymph node cells from CFA-treated mice respond poorly to mitogen or anti-CD3 stimulation (275). Mac-1$^+$ cells purified from CFA-treated NOD mice spleens or from thioglycollate-induced peritoneal exudate cells markedly suppressed splenic T lymphocyte or thymocyte responses to Con A or anti-CD3 (275). In addition, CFA administration to diabetic NOD mice at the time of islet transplantation, prevents graft rejection (276, 277). Although CFA protects against diabetes and islet graft rejection in NOD mice, transfer of spleen cells from CFA-protected mice to healthy syngeneic mice did not protect against disease unless recipient mice received CFA 24 hours after spleen cell transfer (277).

Complete Freund's adjuvant-treated mice are free from intraislet lesions, and peri-islet lesions contain a lower ratio of CD45R/CD45 lymphocytes compared to untreated mice (278). T lymphocytes from destructive islet lesions immunostain for IFN-γ whereas T lymphocytes non-destructive lesions immunostain IL-4 (278). CFA-mediated protection against diabetes is time-dependent and must be given between 4 and 10 weeks of age (279).

Animals treated with *Mycobacterium tuberculosis* or *Mycobacterium bovis* (BCG vaccine) starting at 5 weeks of age are also protected against diabetes and insulitis development (279). Spleen cells or draining lymph node cells from CFA-protected NOD mice could significantly protect against diabetes in untreated mice, and when mixed with spleen cells from acutely diabetic NOD and passively transferred to irradiated non-diabetic NOD mice, delayed the onset of diabetes (279). T lymphocytes, and more precisely, CD4 T lymphocytes appear to be responsible for this protection since depletion of T lymphocytes with anti-Thy 1.2 mAb or with anti-CD4 mAb abrogated protection (279). CFA-mediated protection of NOD mice could be abrogated with two doses of cyclophosphamide suggesting that CFA prevents the induction of effector cells (279). CFA decreases Thy.1$^+$, CD3$^+$ and CD4$^+$ lymphocytes and increases Mac-1$^+$ and Ig$^+$ cells in both spleen and lymph nodes and moderately increases the number of CD4$^+$ CD8$^+$ thymocytes and reduces CD4$^-$ CD8$^-$ thymocytes (279). This indicates that CFA-mediated protection against diabetes and insulitis results in increased numbers of natural suppressor cells, myeloid precursor cells and inhibits the induction of effector cells in the NOD mouse and likely in the BB rat. Furthermore, CFA increases TNF-α and IL-4 production which could immunoregulate autoreactive cells to a state of non-responsiveness.

Poly [I:C] is an inosinic and cytidylic acid-derived polyribonucleotide capable of stimulating IFN-α and IFN-β from macrophages resulting in autocrine activation of these cells (280). Poly [I:C]-treated NOD mice are protected against diabetes and insulitis development and

T lymphocytes from these mice suppress NOD T lymphocytes in MLC (280). However, Poly [I:C]-treated DP or DR rats develop diabetes and insulitis (281). This is in contrast to the suggestion that Poly [I:C] alone is only weakly effective in inducing diabetes in DR rats and accelerating diabetes in DP rats (282-284), and delayed with anti-IFN-α antibody administration (284). Adjuvants such as OK-432 and CFA prevent autoimmune disease in NOD mice and BB rats by altering cell numbers and increasing cytokine production, however, Poly [I:C] has opposite effects NOD mice and BB rats.

CYTOKINES AND DIABETES: Cytokines are polypeptide products released from lymphocytes, macrophages and other immune cells and act in an autocrine, paracrine or endocrine manner to regulate an immune response. Defects within this immunoregulatory communication network could result in autoimmune disease.

INTERLEUKIN 1 (IL-1): Low-dose IL-1β treatment of DP rats starting from 25-35 days of age prevented diabetes and insulitis (285, 286). In contrast, high dose IL-1β treatment of DP rats accelerates diabetes onset and induces thymic hypertrophy (286). One mechanism in which low, but not high dose IL-1 administration may prevent against diabetes development is by stimulating macrophage TNF production.

INTERLEUKIN 2 (IL-2): Interleukin 2 (IL-2) is a T lymphocyte-derived lymphokine which acts as a T lymphocyte growth factor.

BB RATS: IL-2 administration to BB rats significantly increased the incidence of diabetes by 120 days, enhanced insulitis characterized by a massive polymorphonuclear granulocyte infiltration in addition to lymphocytes and macrophages, and also caused inflammation of the exocrine pancreas (287). No significant differences in peripheral blood leukocyte subsets were observed (287). Low dose or high dose IL-2 treatment of DP rats did not accelerate or prevent diabetes development and high dose IL-2 treatment of DR-BB rats was without effect (288). In contrast to these earlier reports, administration of IL-2, while increasing the incidence of, and accelerating diabetes onset, in subline 1 BB rats, prevented diabetes in subline 2 BB rats (289). However, IL-2 treatment enhanced exocrine pancreatic tissue inflammation in both sublines (289). Subline 2 BB rats also demonstrated increased IL-2 inhibitory activity and decreased serum thymosin α_1 and β_4 levels (289).

NOD MICE: IL-2 administration to NOD mice decreased diabetes and increased pancreatic islet insulin levels (280). T lymphocytes from IL-2-treated NOD/Lt mice used as stimulator cells, suppressed syngeneic MLR responses and also acquired suppressor function through increased peritoneal macrophage IL-1 production (280).

IL-2 TRANSGENICS: The importance of IL-2 in diabetes development was demonstrated in β-cells of mice which expressed an IL-2 transgene (290). Whereas the exocrine pancreas of these mice was almost totally destroyed, insulin-containing islets remained intact (290). Adult transgenic mice had extensive peri- and intra-islet infiltrates of small lymphocytes but insignificant levels of IL-2. Severity of the pancreatitis was related to the composition of the infiltrates and amount of IL-2 produced (290). Pancreatitis was similar in mice expressing both islet cell Class I MHC allo-antigen and IL-2 transgenes (290). Mice expressing islet cell Class I MHC allo-antigen, IL-2 and TcR transgenes developed severe

diabetes as early as one week after birth indicating that T lymphocytes can destroy β cells provided that these cells express both IL-2 and Class I MHC allo-antigen (290).

INTERFERONS (IFN): Human pancreases from recent-onset IDDM hyperexpressed Class I MHC and IFN-α molecules and most insulin-staining β cells also stained for IFN-α (292). A role for IFN-α in IDDM was investigated in mice islets expressing a human IFN-α transgene (293). Hybrids of IFN-α transgene-expressing mice backcrossed to CD1 mice became diabetic from 2-24 weeks of age with an incidence of diabetes of greater than 50% and insulitis was composed of a mononuclear infiltrate consisting mainly of CD4 lymphocytes (293). Treatment of these mice with an anti-IFN-α monoclonal antibody significantly reduced the incidence of diabetes (293). These results indicate that directed expression of IFN-α may be associated with diabetes development.

Transplantation of WF islets pre-treated with TGF-β for 7 days into C57BL/6J mice increased islet survival and this was enhanced by anti-IFN-γ mAb treatment (294). Animals transplanted with a second islet graft after TGF-β + anti-IFN-γ treatment also survived (294).

Diabetes was prevented in anti-IFN-γ antibody-treated BB rats and NOD mice (295, 296). Insulitis was decreased and was associated with reduced Class II MHC antigen expression on islet cells and reduced Class I MHC antigen expression on islet and exocrine cells (296, 297). Anti-IFN-γ antibody-treated male NOD mice passively transferred with acute diabetic mice spleen cells were also protected from developing diabetes (296).

IFN-γ TRANSGENICS: Mice expressing β cell IFN-γ transgenes had a lymphocytic infiltration of the pancreas at about 3 weeks, and insulitis and peri-insulitis by 10-20 weeks resulting in the loss of islet integrity and β cells (298). Transgenic mice backcrossed to severe combined immune deficiency (SCID) mice demonstrate that infiltrating T lymphocytes are responsible for islet destruction and also demonstrate that IFN-γ impairs glucose responsiveness of islets (298, 299). Histocompatible islets transplanted under the kidney capsule of diabetic and pre-diabetic mice, and IFN-γ/SCID mice were infiltrated by lymphocytes, histiocytes, plasma cells and fibroblasts resulting in islet disruption and β cell death (298). Both transgenic and non-transgenic mouse lymph node cells primed *in vitro* with irradiated islets were able to kill untreated, but not protease-treated islet cell targets in a CML assay suggesting that cell surface antigen expression on islet cells are necessary for diabetes development (298).

Anti-CD3-treated islets isolated from cyclophosphamide (Cy)-treated NOD mice released IFN-γ and IL-6 (299). Treatment of Cy-treated NOD with an anti-IFN-γ or anti-IL-6 antibody significantly reduced diabetes and insulitis (299). NOD mice were also treated with IFN-γ, TNF-α, or IFN-γ + TNF-α, and those IFN-γ + TNF-α-treated NOD mice displayed a lymphocytic and polymorphonuclear infiltration of the exocrine pancreas and intact insulin-staining islets cells which had increased expression of MHC Class I and Class II antigen cell surface expression (300). This suggests that TNF-γ administration reduced the incidence of diabetes by reducing the degree of insulitis in NOD pancreas.

INTERLEUKIN 10 (IL-10): Interleukin 10 is an anti-inflammatory cytokine produced by Th_1 lymphocytes, B lymphocytes and macrophages (165). Balb/c mice expressing islet cell IL-

10 transgenes developed insulitis starting 4-6 weeks of age characterized by a macrophage infiltration followed at 2-3 months by CD4 and CD8 lymphocytes within the exocrine pancreas and islets of Langerhan (165). Increased intercellular adhesion molecule 1 (ICAM-1) and MHC Class II antigen expression, and increased secretion of von Willebrand factor (vWF), expression of vascular addressins, and leukocyte adhesion molecules were associated with the inflammatory foci (165). However, diabetes is prevented and insulitis reduced in NOD mice administered IL-10 from 9 weeks of age to 25 weeks of age (301). This suggests that IL-10 acts systemically rather than locally to suppress effector cell activity to prevent diabetes and insulitis.

PROSTAGLANDINS: Prostaglandins inhibit the production of IL-2 and IFN-γ from T_{h1} clones without affecting IL-4 and IL-5 production by T_{h2} clones, and suppresses macrophage TNF-α and IL-1 production (302, 303). BB rats treated with PGE_1, or a PGE_1 derivative have a greater incidence of diabetes with earlier onset and significant increases in the absolute numbers of IL-2R$^+$, Class II MHC antigen-positive T_h and $T_{c/s}$ lymphocytes (304).

OTHER FACTORS: Thymosins are a group of a thymic hormones involved in the maturation and differentiation of thymocytes (305). Thymosin fraction 5-treated BB rats are not protected against diabetes development although the W3/25$^+$:OX-8$^+$ cell ratio increases (306). BB rats treated with macrophage-colony stimulating factor (M-CSF) from 40-100 days of age did not reduce the incidence or timing of diabetes onset (307). However, treatment of NOD mice with murine GM-CSF reduced the incidence of diabetes by 50% (270). Cortisone-treated BB rats were also not protected from diabetes development (308). The inability of cortisone to protect against diabetes may be associated with a point mutation in the corticosteroid binding globulin (CBG), reducing corticosteroid binding to CBG by 33% (309).

TUMOUR NECROSIS FACTOR AND DIABETES

TUMOUR NECROSIS FACTORS: The TNF-α and TNF-β (lymphotoxin; LTα) genes are single copy genes of about 3 kilobases (kb) consisting of four exons and three introns closely linked within the MHC with TNF-β 5' to TNF-α (310). Mature TNF-α consists of 157 amino acids (aa; 156 aa in the rat), and gives rise to a 17 kDa protein whereas TNF-β (LTα), is 171 aa (169 aa in the rat), and gives rise to a 25 kDa protein (310). Native TNF-α and TNF-β (LTα) exist as tightly packed trimers of 45 kDa and 66 kDa respectively (310). TNF-α contains a 76 aa long precursor sequence (79 in rat), which serves as an anchor for the non-glycosylated integral membrane-bound protein of 26 kDa, which is cleaved by a serine proteinase to give rise to a 17 kDa released form (310). While human TNF-α exist as 17 kDa and 26 kDa forms, murine TNF-α exist as 18.5 kDa, 21-25 kDa, 27 kDa, 29 kDa, and 32 kDa forms which have been demonstrated to arise from differential (N)-linked glycosylation and sialylation (311-314). However, no biological activities have been attributed to these higher molecular weight forms.

Tumor necrosis factor β (TNF-β or LTα) contains a 34 aa precursor sequence (33 in rat), containing a typical signal peptide sequence of secretory proteins and is processed and released from cells in a manner characteristic of most secreted proteins (310). Recently, a second form of TNF-β, called LTβ is 25-26 kDa protein of 224-244 aa long with a 15-19 aa N-terminal cytoplasmic domain, and can exist as both membrane-bound and released

(trimer) forms (315, 316). LTα and LTβ can form heterotrimers in α_2:β_1 ratio (315, 316). Additional forms of TNF-β (LTα) have been described as 21.5 kDa, 23 kDa, 24 kDa, 26 kDa forms differing in the amount of (N)-linked glycosylation (317). No function has yet been attributed to the higher molecular weight forms of TNF-β (LTα) or the newly described LTβ.

IMMUNOLOGY OF TUMOUR NECROSIS FACTORS: TNF-α is produced mainly by macrophages and monocytes, with lesser amounts produced by T_{h1} CD4 and CD8 T lymphocytes, B lymphocytes, NK cells, lymphokine-activated killer (LAK) cells, mast cells, polymorphonuclear cells, keratinocytes, astrocytes, microglial cells, smooth muscle cells, intestinal paneth cells, some tumours, endothelial cells and mesangial cells (310, 318, 319). The main producers of LT include T_{h1} CD4 and CD8 T lymphocytes, B lymphocytes and astrocytes (310).

Tumour necrosis factor alpha (TNF-α) treatment of mice reduces splenic CD4, CD8 and B lymphocyte numbers and increases splenic monocytes numbers, inhibits T and B lymphocyte proliferation in response to mitogens, NK cell- and CTL-mediated cytotoxicity, without affecting humoral immunity (320). TNF-α is a macrophage maturation/differentiation factor (321), is important in T lymphocyte development (322), and participates in thymic ontogeny by inducing apoptosis of double-negative and single positive thymocytes (323). TNF-α enhances Class II MHC antigen expression on immature lymphoid cells and decreases it on mature lymphoid cells (324), and on bone-marrow derived and splenic macrophages (325). Membrane-bound TNF-α on activated $CD4^+$ lymphocytes is co-stimulatory in IL-4-dependent Ig synthesis by B lymphocytes (326), and in macrophage activation (327). Therefore, *in vivo*, systemic TNF and membrane-bound TNF-α can act to immunomodulate immune responses which may be important in preventing autoimmune disease.

ADMINISTRATION OF TUMOUR NECROSIS FACTOR: Tumour necrosis factor α alone, or in combination with other pro-inflammatory cytokines such as IL-1 and/or IFN-γ has been shown to play a direct role as an effector molecule in islet cell killing (141-149, 155, 157, 158). However, the administration of mitogens such as LPSw or adjuvants such as OK-432 or CFA to NOD mice protect against diabetes development, increase serum TNF levels (269, 270), and release of TNF by splenocytes (275). Simultaneous administration of OK-432 with an anti-TNF-α monoclonal antibody to NOD mice prevents OK-432-mediated protection against diabetes development (269). The same level of protection against diabetes in NOD mice could be obtained with administration of LPSw or TNF-α (311). These results suggest a role for TNF-α in the protection of autoimmune diabetes in the NOD mouse. Administration of TNF-α alone, or with IFN-γ to NOD mice also protected against diabetes and insulitis development with normal-appearing islet containing intact insulin-producing β cells (300). Together, these results suggest that locally produced TNF-α by islet infiltrating macrophages or lymphocytes can play an effector role in β cell killing while systemic increases in TNF-α levels can protect against diabetes by down-regulating effector responses and cellular immune function.

NOD mice or BB rats treated with TNF-α are nearly completely protected against diabetes development and insulitis (270, 328, 329). Spleen cell subsets from BB rats are significantly less cytolytic against YAC-1 and RINm5F cells (329). Transplantation of WF

islets into C57BL/6J mice are protected from rejection if TNF-α is administered from 3-60 days post-transplantation (330). In contrast, administration of anti-TNF-α mAb significantly increases insulitis in NOD mice (331). However, TNF-α and anti-TNF-α antibodies may display dual personalities since administration of anti-TNF-α antibody from birth until 3 weeks of age prevents diabetes while administration of TNF-α during the same period accelerates disease development by about 4 weeks and increases the incidence to 100% (332). Furthermore, passive transfer of diabetes is prevented in young non-diabetic NOD mice given diabetic NOD mouse splenocytes if the recipients are given TNF-α 3 days after transfer (333).

Adminstration of LT (TNF-β) also significantly reduces diabetes and insulitis in NOD mice and BB rats (334, 335), and increases the SMLR by NOD spleen cells (334). This suggests that TNF-α and LT (TNF-β) production may be deficient in diabetes-susceptible animals since stimulated serum and peritoneal exudate cell TNF/LT levels in NOD mice are severely depressed compared to other mouse strains (270, 328). Stimulated TNF levels by BB rat spleen cells are also depressed whereas stimulated BB rat serum TNF levels are similar to Wistar rats (335). In contrast, it has been reported recently that BB rat peritoneal and BM-derived macrophage TNF production is upregulated in comparison to DR and WF rats (336). However, these DP rats were primed with *Corynebacterium parvum*, a potent and chronic macrophage activator, 4 days prior to harvest (336). In addition, cyclophosphamide treatment of DP rats also upregulates peritoneal macrophage TNF production (63).

Upregulated TNF production by DP rat peritoneal macrophages can be suppressed in culture by the addition of Wistar peritoneal macrophages or supernatants (337). The suppressor factor which was determined to be PGE_2, is significantly depressed in *Corynebacterium parvum*-stimulated DP rats (337, 338). This is contrast to another report that demonstrates that resident splenic adherent cells from DP rats produce TNF levels comparable to WF, NEDH and Lewis rats whereas TNF production by DP peritoneal macrophages were normal after 4 hours of culture but severely deficient at 18 hours of culture (339). Furthermore, serum TNF levels is increased after stimulation *in vivo* only in newly diabetic or long-term diabetic BB rats (340). This suggests that TNF production in BB rats may be dysregulated rather than deficient or excessive. This suggests that other factors such as PGE may be important in diabetes development.

TUMOUR NECROSIS FACTOR POLYMORPHISMS AND DIABETES: BB RAT TNF-α GENES: The *TNFA* locus of the rat maps between RT1.*D* and RT.1*E* (341) and MHC Class II genes and both are thought to be involved in BB rat diabetes development (14, 22). Restriction fragment length polymorphisms within the TNF-α gene may correlate to the proneness in developing IDDM. While there are no polymorphic differences between DP, DR and Wistar rats, two polymorphisms are found to exist in a dinucleotide repeat found in the TNF promoter region in DP rats that is thought to be associated with dysregulated TNF-α production (63). Crosses between DP and LEW.1A rats resulted in TNF-α dysregulation in those rats which carried either a homozygous or heterozygous BB rat TNF allele (63). TNF gene dysregulation is not due to mutations in NF-kB2/CK-1, NF-kB2b, NF-kB3, NF-kB4/CK-1, and Y-BOX regulatory elements in the TNF gene promoter region and are not different from other rat strains (337, 338).

NOD MOUSE TNF-α GENES: Although there are interallelic upstream regulatory polymorphisms in the microsatellite repeat of the TNF-α gene in NOD mice, they are not different from non-obese nondiabetic (NON) mice (342). However, NOD mice do show a unique heat shock protein 70 (HSP70) haplotype suggesting that this protein may be associated with NOD diabetes (342).

HUMAN TNF-α GENES: There are three distinct TNF microsatellite polymorphisms in the human TNF gene and include TNFa, TNFb, and TNFc (343). High TNF-α release by monocytes correlates with the TNFa2 ,a9, a13, and TNFc2 alleles, and HLA-DR3 and -DR4 haplotypes, whereas low TNF-α release correlates with TNFa1 and a6 alleles, and HLA-DR2 and -DR5 haplotypes (343). DR4-associated high TNF-α release alleles were more frequently found in IDDM patients compared to controls (344). This suggests that in rat, mouse and man, polymorphisms in TNF loci may play a role in determining susceptibility to diabetes. In addition, HSP70 may also be a determining factor in NOD mouse diabetes development.

TUMOUR NECROSIS FACTOR ALPHA AND BETA TRANSGENICS ANIMALS: At 3 weeks of age, mice containing islet cell-directed TNF-α transgene expression had an infiltration of $Thy1^+$ $CD4^+$ or $Thy1^+$ $CD8^+$ T lymphocytes of which few were $IL\text{-}2R^+$, sIg^+ B lymphocytes and a few peripherally located macrophages, without changes in fasting glycemias (345). Pancreases of hybrid mice derived from transgenic mice crossed with SCID mice did not show any detectable lymphocytic infiltration (345). Cyclophosphamide-treated transgenics did not develop diabetes, whereas some anti-CD3-treated transgenic mice had a transient and mild hypoglycaemia (345). Some anti-CD3-treated transgenic mice also treated with IL-2, rIL-1β, or IFN-γ showed a lymphocytic infiltration without β cell damage or hyperglycaemia (345). Furthermore, no increase in diabetes development was observed in streptozotocin-treated transgenic mice or in mice expressing islet cell-directed TNF-α and LCMV-GP transgenes, even after IFN-γ treatment (345).

Mice expressing β cell-directed TNF-β transgenes and TNF-β mRNA and protein, produced insulitis which was characterized both $CD4^+$ and $CD8^+$ lymphocytes and $B220^+$ IgM^+ lymphocytes, without producing overt diabetes (346). In a different strain of mice expressing islet cell-directed TNF-α transgenes, TNF-α mRNA and protein was expressed by islets and insulitis present throughout the islet was characterized by $CD4^+$, $CD8^+$, $B220^+/IgM^+$ B cells and some macrophages (347). Although the infiltrating cell phenotypes were the same, numbers were 100-fold greater in TNF-α transgenic compared to the TNF-β transgenic mice (347). Endothelial cells in the islets of TNF-α and TNF-β transgenics expressed vascular cell adhesion molecule-1 (VCAM-1), ICAM-1 and Class II MHC antigens (347). While insulitis in TNF-α and peri-insulitis in F_1 TNF-β mice on a NOD background were similar to the original transgenics, none of these F_1 progeny became diabetic (347). In contrast, 2 of 4 double mice expressing islet-directed TNF-α and LCMV-GP transgenes, and infected with vaccinia virus glycoprotein (vacc-gp) to increase lymphocyte traffic, became diabetic (348). This suggests that islet cell-directed TNF-α/β expression can recruit lymphocytes resulting in different patterns of insulitis, a second event is necessary for diabetes development. This second event may be inappropriate or cryptic antigen expression on the islet cell itself, and together, these events lead to insulitis and diabetes development.

STATEMENT OF THE PROBLEM

Insulin-dependent diabetes mellitus (IDDM) is a polygenic autoimmune disease involving macrophages and lymphocytes, and their secreted protein products, cytokines. Together, macrophages and lymphocytes directly, or through their cytokines specifically and selectively destroy the insulin-producing pancreatic beta cells of the islets of Langerhans.

A mononuclear infiltrate comprised mostly of Class I and Class II major histocompatibility complex (MHC)-positive macrophages is first observed in the islets of Langerhans just prior to, and during the earliest stages disease onset. Later, Class II MHC-positive lymphocytes are found in the lesions. This implies an important role for the macrophage in diabetes. It has also been demonstrated *in vitro* that macrophage cytokines, namely, interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α) alone, or in synergy with a lymphocyte cytokine, interferon gamma (IFN-γ), results in pancreatic beta cell killing.

More recently however, it has been demonstrated that administration of macrophage activators or chronic administration of TNF-α to diabetes-prone BB rats and non-obese diabetic (NOD) mice protects against the development of insulin-dependent diabetes mellitus (IDDM). Macrophages derived from NOD mice produce lower levels of TNF-α after stimulation *in vitro*. This suggests that deficient TNF-α production by macrophages may participate in disease development, and more importantly, TNF-α may be a beneficial cytokine by protecting the insulin-producing beta cell from activated autoreactive T lymphocytes.

This Doctor of Philosophy in Medical Sciences thesis research was designed to evaluate the capacity of different lymphoid cells to produce tumour necrosis factor. The objective of these studies was to identify whether tumour necrosis factor production is dysregulated in the BB rat model of IDDM, which cells produce dysregulated amounts of TNF, and whether non-specific immunostimulation with complete Freund's adjuvant (CFA) corrects this dysregulation.

Experiments were done in following ways: TNF levels were measured in culture supernatants and cell cytosol of peritoneal cells and splenocytes from BB rats stimulated *in vivo*, and in culture supernatants, cell cytosol, and membrane fractions by peritoneal cells, splenocytes, and thymocytes from saline- or CFA-treated BB rats stimulated in culture. Studies were extended to determine whether TNF levels in peritoneal cells from adult non-diabetic diabetes-prone BB rats were sufficient to prevent diabetes.

Additional experiments were done to characterize deficient TNF production in BB rat peritoneal cells. Two-colour flow cytometry was used to determine whether a deficiency exists in lipopolysaccharide and CD14 receptors expression on peritoneal cells and whether CFA corrects this deficiency. In addition, TNF levels were measured in peritoneal cells after stimulation with cytokines or with protein kinase and calcium channel agonists to determine whether defects exist in signal transduction pathways or whether TNF production in these cells was suppressed by other factors such as prostaglandins.

Studies were also done to determine whether CFA protects against diabetes development in BB rats through increased TNF production. Therefore, a polyclonal antibody to TNF-α was chronically administered in order to reverse the CFA-mediated protection.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Diabetes-prone (DP) and diabetes-resistant (DR) sublines of Wistar-derived BB rats were obtained directly from, or in house from breeding pairs obtained from the NIH contract colony (BB/Wor) at the University of Massachusetts Medical School, Worcester, Massachusetts (Dr. A. A. Like). All DP and DR rats are viral antibody-free (VAF) and were housed and maintained under VAF conditions at the University of Alberta Health Sciences Laboratory Animal Services (HSLAS), were weaned at 17-21 days of age, and male and female rats from each litter were divided randomly into different experimental groups. Older DP and DR (ages 182-220 days) failed to develop diabetes and were normoglycaemic. Wistar Furth (WF) and LEWIS rats (Charles River, CA.) are VAF and were housed and maintained under VAF conditions. BB (DP and DR), and WF are *u* haplotype and LEWIS rats are *l* haplotype at RT1 (3).

TREATMENT WITH COMPLETE FREUND'S ADJUVANT (CFA)

Diabetes-prone and DR rats were treated with CFA as described (273). Briefly, DP and DR rats (ages 22-28 days) were injected intraperitoneally with 100 uL/50 g body weight of either CFA (Difco Laboratories, Detroit, MI.) or sterile saline (PBS). Rats were used in experiments 10-14 days later.

PREPARATION OF CELLS

Rats were anaesthetized by peritoneal or subcutaneous injection of Somnotol (50 mg/kg; M.T.C. Pharmaceuticals, Cambridge, ON.) and peritoneal cells removed under aseptic conditions. Fifty mL of ice-cold sterile Ca^{2+} and Mg^{2+} -free phosphate buffered saline (PBS) containing 22 mM ethanolamine disodium tetra-acetic acid, (EDTA, Sigma Chemicals, St. Louis, MO.), or Dulbecco's PBS (D'PBS) was injected into the peritoneal cavity using an 18 gauge needle (Becton Dickinson, Rutherford, N.J.), the peritoneum was gently massaged for 2-3 minutes, the contents were slowly withdrawn, placed in a 50 mL sterile conical centrifuge tube (Becton Dickinson) and stored on ice until needed. Peritoneal cells were collected from untreated, PBS-, or CFA-treated DP and DR rats, ages 23-45 days of age for young rats (DR-YOUNG and DP-YOUNG), from ages 182-220 days of age for old rats (DR-OLD and DP-OLD), from WF and LEWIS rats.

Red blood cells were lysed by hypotonic shock with an ammonium chloride solution (ACK) consisting of 1.0 mL of ACK solution (8.29 g NH_4Cl [Fisher, Edmonton, AB.], 1.00 g $KHCO_3$ [Fisher], 0.367 g EDTA, brought to a final volume of one litre with double distilled water, adjusted to pH = 7.4), and filter-sterilized (349). One mL of ACK solution was added to a soft pellet of peritoneal cells obtained from one lavage, vortexed, and incubated at room temperature for 2 minutes. Forty-nine mL of ice-cold Hank's buffered saline solution (HBSS; Gibco, Burlington, ON.) was added and the cells centrifuged at 1400 rpm for 10 minutes at 4°C, the supernatant aspirated, and the washing procedure repeated twice more to remove remaining red cell membranes. After the last wash, peritoneal cells were resuspended in Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (Gibco) and 0.05 g/mL gentamycin (complete medium) and stored on ice.

Peritoneal cell number and viability was determined using the trypan blue exclusion method and always exceeded 95%. In some experiments, peritoneal macrophages were enriched (≥ 75% purity) from peritoneal cells by centrifugation on a Ficoll-Hypaque (Pharmacia, Pointe Claire, QC.) density gradient (350).

Splenectomies and thymectomies were performed using standard aseptic dissecting techniques. Rats were anaesthetized as above. Spleens or thymuses were excised and placed in sterile 10 mL round bottom tubes (Fisher) containing ice-cold sterile HBSS. A single cell suspension was prepared by passing the spleen through a stainless steel sieve (Sigma). Tissue fragments and debris were allowed to settle out and the cell suspension was transferred to a sterile 50 mL conical tube and centrifuged at 1600 rpm for 10 minutes at 4°C. Red blood cells were removed as described above. Cell number and viability was determined using the trypan blue exclusion method and always exceeded 95%.

IN VITRO *ACTIVATION OF CELLS*

Peritoneal cells were incubated for 1.5, 3, 6, or 18 hours in complete medium alone, in medium containing recombinant murine interferon-gamma (IFN-γ;100 U/mL of 10^5 U, specific activity 4.7 X 10^6 U/mg; Genentech, South San Francisco, CA.), endotoxin-inactivated lipopolysaccharide-*Salmonella minnesota* (LPS; 0.5 ug/mL; Sigma), IFN-γ + LPS, recombinant human interleukin-2 (IL-2; 1000 U/mL from a stock of 5000 U/ml, Genzyme, Boston, MA.), IL-2 + IFN-γ, IL-2 + LPS, phorbol, 12-myristate, 13-acetate (PMA; 10 ng/mL; Sigma), A23187 (5 uM; Sigma), PMA + A23187, indomethacin (5 ug/mL; Sigma) or indomethacin + LPS in 24-well tissue culture flat-bottom plates (Corning Glass Works, Corning, NY.) in a humidified incubator at 37°C and gassed with 95% air and 5% CO_2. After incubation, media were collected and frozen at -70°C until assay for TNF content. An identical volume of fresh complete medium was added to each well and the plate frozen at -70°C until assay for cytosolic and membrane TNF content.

Spleen cells were incubated for 1.5, 3, 6, or 18 hours in complete medium alone, in medium containing IFN-γ (100 U/mL), LPS (0.5 ug/mL), IFN-γ + LPS, concanavalin A (Con A; 5 ug/mL; Sigma) or phytohemagglutinin (PHA; 4 ug/mL; Sigma) in 24 well tissue-culture flat-bottom plates (Corning) in a humidified incubator at 37°C and gassed with 95% air and 5% CO_2. After incubation, media were collected and frozen at -70°C until assay for TNF content. An identical volume of fresh complete medium was added to each well and the plate frozen at -70°C until assay for cytosolic and membrane TNF content.

Thymocytes were incubated for 1.5, 3, 6, or 18 hours in complete medium alone, in medium containing IFN-γ (100 U/mL), LPS (0.5 ug/mL), IFN-γ + LPS, concanavalin A (Con A; 5 ug/mL; Sigma) or phytohemagglutinin (PHA; 4 ug/mL; Sigma) in 6 well tissue culture flat-bottom plates (Corning) in a humidified incubator at 37°C and gassed with 95% air and 5% CO_2. After incubation, media were collected and frozen at -70°C until assay for TNF content. An identical volume of fresh complete medium was added per well and the plate frozen at -70°C until assay for cytosolic and membrane TNF content.

PREPARATION OF CELLULAR COMPONENTS

Cytosolic and membrane fractions of peritoneal and spleen cells, and thymocytes were prepared by rapid thawing at room temperature. The plates were centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatants containing the cytosolic fractions were

carefully removed to avoid resuspending the cell membranes and placed into sterile 4 mL tubes (Fisher) and placed on ice. The volume of medium that was removed was replaced with the same volume of ice-cold complete medium, and the cell membranes removed from the well bottoms by repeated scraping and aspiration. Membrane fractions were transferred to sterile 4 mL round bottom tubes and placed on ice until needed.

STIMULATION OF RATS BY INJECTION IN VIVO

Three methods were used to stimulate *in vivo* TNF release into the serum and production by peritoneal and spleen cells.

A) In some experiments, DP, DR, WF and LEWIS rats (ages 25-40 days) were injected intravenously (tail vein) with (a) 1.0 mL sterile D'PBS followed 2 hours later by 1.0 mL D'PBS, (b) 1.0 mL D'PBS followed 2 hours later by 1.0 mL D'PBS containing LPS (500 ug), (c) 1.0 mL D'PBS containing IFN-γ (10^5 U) followed 2 hours later by 1.0 mL D'PBS, or (d) 1.0 mL D'PBS containing IFN-γ (10^5 U) followed 2 hours later by 1.0 mL D'PBS containing LPS (500 ug). Two hours after the last injection, animals were anaesthetized and blood withdrawn by cardiac puncture using an 18 gauge needle attached to a 10 mL syringe. The blood was transferred to a sterile 10 mL round bottom tube (Fisher) and incubated at room temperature for 20 minutes to allow clotting and on ice for an additional 30 minutes to allow clot contraction. Serum was transferred to sterile 1.5 mL centrifuge tubes (Fisher) and frozen at -70°C until assay for serum TNF and corticosterone content. Peritoneal cells and spleens were removed and cells were prepared by the methods described.

B) In other experiments, DP, DR, WF and LEWIS rats were (ages 25-40 days) were injected intravenously (tail vein) with 1.0 mL D'PBS or D'PBS containing LPS (500 ug). After 0.5, 1.0, 1.5 and 2.0 hours, rats were anaesthetized and blood withdrawn by cardiac puncture and processed as described above. Peritoneal cells were obtained and prepared as described above.

C) Finally, DP, DR, CFA-treated DP and DR, WF and LEWIS rats were (ages 25-40 days) were injected intravenously (tail vein) with 1.0 mL D'PBS containing LPS (500 ug). After 0.5, 1.0, 1.5 and 2.0 hours, rats were decapitated and bled into 10 mL Vacutainer tubes (Becton Dickinson), incubated at room temperature for 20 minutes followed by 30 minutes on ice. Tubes were centrifuged at 1800 rpm for 30 minutes at 4°C, the serum removed and sterilized by filter centrifugation at 14, 000 rpm for 1 minute using 1.5 mL centrifuge tubes containing a 0.22 um filter attachment (Costar; Cambridge, MA.). Sterile sera were aliquoted into sterile 1.5 mL centrifuge tubes and frozen at -70°C until assay for TNF or corticosterone content. Peritoneal cells were obtained and prepared as described above. Sera and peritoneal cells prepared from unmanipulated animals served as 0 hour controls.

TUMOUR NECROSIS FACTOR (TNF) ASSAY

TNF concentrations in cell incubation media were determined by a highly sensitive cytolytic bioassay (murine TNF-α detection limit 0.2 pg/mL and TNF-β limit, 0.05 pg/mL) specific for TNF, by using an L929 subclone, L929-8 (351). L929-8 cells were grown to log phase in complete medium, the monolayer washed once with warm D'PBS, dispersed in Ca^{2+} and Mg^{2+} -free PBS containing 0.05% (*w/v*) trypsin and 0.02% (*w/v*) EDTA (Gibco)

washed, and plated at a density of 5 X 10^4 cells/50 uL/well in complete medium supplemented with 2 ug/mL actinomycin D (Sigma) in 96-well tissue culture flat-bottom plates (Corning). After 1 hour of pre-incubation at 37°C, serial dilutions of sample media (50 uL) were added to the L929-8 cells. For sera, the starting dilution was 1:32 since lower dilutions affected the growth and response of L929-8 cells to TNF (personal observation). Recombinant murine TNF-α (specific activity 1.2 X 10^7 U/mg, Genentech) standards were included in each assay plate. The plates were incubated for an additional 18 hours at 37°C, then 50 uL of 0.05% (*w/v*) neutral red (Sigma) in normal saline was added to each well. The plates were incubated for an additional 2 hours at 37°C, then the media were decanted, the plates washed twice with PBS and 100 uL of 50% (*v/v*) ethanol in 0.05 M sodium phosphate was added. The optical density (O.D.) was determined spectrophotometrically (reference wavelength = 650 nm; sample wavelength = 450 nm) on a Molecular Devices *Vmax* kinetic microplate reader (Molecular Devices, Menlo Park, CA.). TNF concentrations were calculated from the O.D. read against the TNF standard curve by extrapolation from a log-logit fit of the standards using the plate reader internal analysis program. All samples (released, cytosol, membrane, sera) were assayed for TNF content within 2 weeks of the experiment.

INDUCTION OF TOLERANCE TO IMMUNOGLOBULINS (Ig) IN NEONATAL RATS

Diabetes-prone and DR rats were bred at the University of Alberta HSLAS. Within 24 hours of birth, new-born pups were tolerized by intraperitoneal injection of 2 mg/pup de-aggregated rabbit Ig or sheep Ig (352). De-aggregated and aggregated fractions of whole rabbit Ig (19.8 mg/mL; Pierce, PDI Bioscience, Edmonton, AB.) or whole sheep Ig (Jackson Immuno Research, West Grove, PA.) were prepared by ultracentrifugation in a 8M Ultracentrifuge (Beckman, Palo Alto, CA.) at 100, 000 X *g* for 3 hours at 4°C. The upper third of the supernatant containing the de-aggregated immunoglobulin (Ig) was removed, filter sterilized and stored at 4°C until needed (353).

QUANTITATION OF RABBIT IMMUNOGLOBULINS (Ig) BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Immulon 3 96 well plates (Dynatech Laboratories, Chantilly, VA.) were coated with 5 ug/mL of a goat anti-rabbit Ig (1.6 mg/mL; Accurate Chemical Co., Westbury, NY.) in PBS pH = 7.4, covered, and incubated in a humidified incubator at 37°C for 30 minutes. The coating antibody was decanted and non-specific binding sites were blocked by adding 100 uL/well of PBS-20% FBS and incubated for an additional 10 minutes in a humidified incubator at 37°C. The PBS/FBS was decanted and the plate was washed 4 times with 200 uL/well PBS-0.05% Tween-20 (Sigma). Serial dilutions (50 uL/well) of samples (PBS pH = 7.4 + 8% [*v/v*] FBS) were added to the plates and incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. Fifty uL/well of a 1 ug/mL second antibody (0.7 mg/mL biotinylated swine anti-rabbit Ig; Accurate; in PBS pH = 7.4, 1% [*v/v*] bovine serum albumin (BSA; Sigma], 0.05% [*v/v*] Tween-20) was added to the wells and the plate incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. Seventy-five uL/well of a 0.5 ug/mL streptavidin-horseradish peroxidase (Biomeda, Foster City, CA.; prepared in PBS pH = 7.4, 1% [*v/v*] BSA, 0.05% [*v/v*] Tween-20) was added to each well and the plate incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted

and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. After the last wash, 100 uL of a 1 mg/mL solution of 2,2'-Azino-*bis*-[3-ethylbenthiazoline-6-sulfonic acid](ABTS; Sigma) prepared in 11 mL of $Na_2HPO_4 \cdot 7H_20$ (53 g/L; Fisher), 14 mL of citric acid (21 g/L; Fisher), 5 uL hydrogen peroxide (30% [*v/v*], Fisher) and 25 mL distilled water. The plate was developed at room temperature and the reaction was stopped by adding 50 uL/well of citric acid (42 g/L). Serial dilutions of normal rabbit Ig (20 mg/mL; Accurate) was used as a standard control antibody and were included in each plate. Other controls included the omission of the coating antibody, the second antibody, streptavidin-horseradish peroxidase, or ABTS. The O.D. were determined spectrophotometrically (sample wavelength = 405 nm and a reference wavelength = 490 nm) using a microplate reader (Molecular Devices). Immunoglobulin concentrations in the samples were determined by extrapolation from a log-logit fit of the standards using the plate reader internal analysis program.

PRODUCTION OF RABBIT ANTI-MURINE TUMOUR NECROSIS FACTOR-α POLYCLONAL ANTISERA

Rabbits were immunized by the method described (354). The immunization procedure conforms to University of Alberta HSLAS and Canadian Council on Animal Care guidelines and was approved by the University of Alberta Bioethics Review Committee. Viral antigen-free New Zealand White rabbits of about 2 kg body weight (Charles River) were immunized intramuscularly with 165 ug recombinant murine TNF-α in CFA in a volume of 0.65 mL and a control rabbit was immunized intramuscularly with 0.65 mL of incomplete Freund's adjuvant (IFA; Difco). Rabbits were again immunized intramuscularly with 165 ug recombinant murine TNF-α in CFA in a volume of 0.65 mL 9-13 days later. Sixty days after the second immunization, rabbits were immunized subcutaneously at 5 different sites with 150 ug TNF-α in IFA (30 uL/site). Five days after the second immunization and 8 days after the third immunization, rabbits were bled and sera prepared to test rabbit antiserum neutralizing activity against TNF-α in the L929-8 TNF bioassay. Twenty-four hours prior to exsanguination, rabbits were fasted. Rabbits were anaesthetized with a Rompun Cocktail [(40 mg/kg Ketamine (Ayerst, St. Laurent, QC.), 8 mg/kg Rompun (Bayvet, Etobicobe, ON.), and 0.5 mg/kg Atravet (Ayerst)] and exsanguinated. After exsanguination, the rabbits were humanely sacrificed with a pentobarbital (M.T.C. Pharmaceuticals) overdose.

DETERMINATION OF TNF-α NEUTRALIZING ACTIVITY IN RABBIT SERA

Rabbit sera prepared prior to immunization (pre-immune bleed), after the second and third test bleeds and after exsanguination were used to determine specific neutralizing activity against TNF-α in the L929-8 TNF bioassay. Briefly, L929-8 cells were prepared as described for the TNF assay. Serial dilutions of the antisera (30 uL) from individual rabbits were added to wells containing 8 or 50 pg/mL (30 uL) recombinant murine TNF-α. TNF and antiserum were mixed directly in the wells of a 96 well tissue culture plate and incubated for 1 hour in a humidified incubator at 37°C. After 1 hour, 50 uL volumes of the TNF/test antisera mixture were added to wells containing L929-8 cells, and the plates incubated for an additional 18 hours. Standards consisted of 5, 2, 1, 0.5, 0.2, 0.1 and 0.001 pg/mL TNF-α and the control consisted of cells alone and were included in each assay plate. The O.D. were determined spectrophotometrically (sample wavelength = 405 nm and a reference wavelength = 490 nm) using a microplate reader (Molecular Devices). The O.D. from the standard curve were read and converted to percent viability.

Neutralizing activity were read as O.D. values and percent viability extrapolated from the standard curve. Neutralizing activities were expressed as titers (1/dilution).

AMMONIUM SULPHATE PRECIPITATION OF IMMUNOGLOBULINS (Ig) FROM RABBIT SERA

Immunoglobulin semi-purification by ammonium sulphate, $(NH_4)_2SO_4$, precipitation was done using standard techniques (355). Rabbit serum was poured into a sterile 500 mL glass beaker (Corning), containing a sterile teflon-coated stir bar (Fisher). The beaker was placed on a stir plate and while stirring gently allowed to cool to 4°C. Analytical grade $(NH_4)_2SO_4$, (Mallinckrodt GenAr, Pointe Claire, QC.) was slowly added over a 6 hour period with constant stirring until 33% saturation (1.3 M) was obtained. The precipitated serum was stirred gently overnight at 4°C, the slurry transferred to sterile round bottom centrifuge tubes (Fisher) and centrifuged at 10, 000 X *g* for 30 minutes at 4°C. The supernatant was decanted and the precipitate dissolved in about one-third the original serum volume in sterile saline, transferred to triple-washed, sterile dialysis tubing with a molecular weight cutoff of 50, 000 (Spectra, Fisher) and dialysed against sterile PBS. Dialysis was done with constant stirring at 4°C over a 3 day period with 2 changes of buffer/day. Typically, 50 mL volumes of sample were dialysed against 10 L PBS, changed twice daily for 3 days.

DETECTION OF RABBIT ANTI-TNF IMMUNOGLOBULINS IN $(NH_4)_2SO_4$ PRECIPITATED RABBIT SERA AND IN RAT SERA BY ELISA

The method used to detect anti-TNF-α Ig in $(NH_4)_2SO_4$ precipitated rabbit sera and in rat sera is similar to that described above for quantitation of rabbit Ig except plates were coated with 50 uL (25 ng/mL murine TNF-α in PBS pH = 7.4), covered, and incubated in a humidified incubator at 37°C for 30 minutes. Supernatants were decanted and non-specific binding sites were blocked by adding 100 uL/well of PBS-20% FBS and incubated for an additional 10 minutes in a humidified incubator at 37°C. The PBS/FBS was decanted and the plate was washed 4 times with 200 uL/well PBS-0.05% Tween-20 (Sigma). Serial dilutions (50 uL/well) of rabbit or rat sera and a standard rabbit polyclonal antisera to murine TNF-α prepared in PBS (pH = 7.4 + 8% [*v/v*] FBS) were added to the plates and incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. Fifty uL/well of a 1 ug/mL second antibody (0.7 mg/mL biotinylated swine anti-rabbit Ig; Accurate; in PBS pH = 7.4, 1% [*v/v*] bovine serum albumin [BSA; Sigma], 0.05% [*v/v*] Tween-20) was added to the wells and the plate incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. Seventy-five uL/well of a 0.5 ug/mL streptavidin-horseradish peroxidase (Biomeda; prepared in PBS pH = 7.4, 1% [*v/v*] BSA, 0.05% [*v/v*] Tween-20) was added to each well and the plate incubated for 30 minutes in a humidified incubator at 37°C. After incubation, supernatants were decanted and the plate was washed 4 times with 200 uL/well with PBS-0.05% Tween-20. After the last wash, 100 uL of a 1 mg/mL solution of 2,2'-Azino-*bis*-[3-ethylbenthiazoline-6-sulfonic acid](ABTS; Sigma) prepared in 11 mL of $Na_2HPO_4 \cdot 7H_20$ (53 g/L; Fisher), 14 mL of citric acid (21 g/L; Fisher), 5 uL hydrogen peroxide (30% [*v/v*]) and 25 mL distilled water. The plate was developed at room temperature and the reaction was stopped by adding 50 uL/well of citric acid (42 g/L). Controls included the omission of the coating antigen, the second antibody, streptavidin-horseradish peroxidase, or ABTS. The O.D. were

determined spectrophotometrically (sample wavelength = 405 nm and a reference wavelength = 490 nm) using a Molecular Devices microplate reader (Molecular Devices). Anti-TNF-α antibody concentration in the samples were estimated from the standard rabbit polyclonal anti-TNF-α antibody by extrapolation from the x-intercept of the dilution factor ($\log_{10}$) *vs.* O.D. program.

TREATMENT OF BB RATS WITH RECOMBINANT TUMOUR NECROSIS FACTOR-α
BB rats were treated with TNF-α as described (329). Nineteen DP rats were treated daily by intraperitoneal injection with 200 uL recombinant human TNF-α (5×10^4 U; specific activity 4.7×10^6 U/mg; kindly provided by Genentech, South San Francisco, CA.), prepared in PBS containing 1% (*v/v*) DP rat serum and 18 DP rats were treated with PBS containing 1% (*v/v*) DP rat serum from 50-110 days of age. Rats were monitored daily for glucosuria and positive rats were confirmed by measuring blood glucose levels for hyperglycaemia. Rats having blood glucose levels ≥ 170 mg/dL were considered diabetic.

TREATMENT OF RATS WITH A POLYCLONAL ANTISERUM TO TUMOUR NECROSIS FACTOR-α
The effect of anti-TNF antisera on diabetes development in PBS-treated and CFA-treated DP rats was tested by the following three methods. New-born rats were tolerized to rabbit or sheep immunoglobulin as described above.

METHOD 1: 14 DP rats were treated once intraperitoneally with 100 uL of sterile PBS and followed to 120 days for diabetes development; 13 DP rats were treated once intraperitoneally with 100 uL of CFA and followed to 120 days for diabetes development; one group of 14 DP rats were treated once intraperitoneally with 100 uL of CFA and then 3 times/week intraperitoneally with 300 uL of a semi-purified [$(NH_4)_2SO_4$-precipitated) rabbit anti-murine TNF-α antisera from 26 days of age until 120 days; and one group of 18 DP rats were treated once intraperitoneally with 100 uL of CFA and then 3 times/week intraperitoneally with 300 uL of a semi-purified [$(NH_4)_2SO_4$-precipitated) normal rabbit serum from 26 days of age until 120 days of age. Rats were monitored daily for glucosuria and positive rats were confirmed by measuring blood glucose levels for hyperglycaemia. Rats having blood glucose levels ≥ 170 mg/dL were considered diabetic.

METHOD 2: 12 DP rats were treated intraperitoneally with 100 uL of sterile PBS 3 times/ week from 26 days of age until 110 days of age; one group of 14 DP rats were treated intraperitoneally with 200 uL of normal sheep serum 3 times/week from 26 days of age until 110 days of age; and one group of 17 DP rats were treated intraperitoneally with 200 uL sheep anti-murine TNF-α antisera as described elsewhere (356) 3 times/week from 26 until 110 days of age. Rats were monitored daily for glucosuria and positive rats were confirmed by measuring blood glucose levels for hyperglycaemia. Rats having blood glucose levels ≥ 170 mg/dL were considered diabetic.

METHOD 3: 10 DP rats were treated once intraperitoneally with 100 uL of sterile PBS at 26 days of age and followed to 110 days of age for diabetes development; 48 DP rats were treated once intraperitoneally with 100 uL of CFA at 26 days of age and 17 became diabetic by 81 days of age; at 81 days of age, one group of 9 DP rats were treated 3 times/week intraperitoneally with 300 uL of a normal sheep serum until 110 days of age; and at 81 days of age, one group of 12 DP rats were treated 3 times/week

intraperitoneally with 300 uL of a sheep anti-murine TNF-α antisera until 110 days of age. The remaining 10 CFA-treated DP rats were followed to 110 days of age for diabetes development. Rats were monitored daily for glucosuria and positive rats were confirmed by measuring blood glucose levels for hyperglycaemia. Rats having blood glucose levels ≥ 170 mg/dL were considered diabetic.

RADIOIMMUNOASSAY OF PROSTAGLANDIN E_2 (PGE_2)

Samples that were assayed for TNF levels were submitted to Dr. D. W. Sadowsky, Department of Obstetrics and Gynaecology, Perinatal Research Centre, University of Alberta for determination of PGE_2 levels. Quantitation of PGE_2 was performed using a specific radioimmunoassay (357). Briefly, non-extracted samples, standards (PGE_2; Cayman Chemical, Ann Arbor, MI), antibody (anti-PGE_2 used a 1:4500; a kind gift from T.G. Kennedy, London, ON.) and trace ([5, 6, 8, 11, 12, 14, 15-N-^{3}H]-PGE_2, specific activity 140 mCi/mmol; Amersham Canada, Oakville, ON.) were added to appropriate tubes and were vortexed. Samples were incubated for 30 minutes at room temperature and for an additional 3 hours at 4°C. Bound and free PGE_2 were separated by the addition of 2 mg/tube/15 minutes of dextran-coated charcoal. After incubation, the tubes were centrifuged using Sep-Pak C18 cartridges (Waters-Millipore; Milford, MA.), and 3 ml of liquid scintillation fluid (OptiPhase 'HiSafe' 3 (LKB Scintillation Products (Loughborough, England) was added to the bound fraction. The tubes were counted on the Beckman LS5000TD liquid scintillation counter and the results were analyzed using its internal RIA program. The assay has a sensitivity, defined as greater than two standard deviations from Bmax, of less than 5 pg, an inter-assay coefficient of variation of 5% at 500 pg, and an intra-assay coefficient of variation of 4% at 500 pg. Blank binding at this dilution of antibody was 40-50%, while the nonspecific binding was <4% of the total radioactivity added to each RIA tube.

RADIOIMMUNASSAY OF SERUM CORTICOSTERONE

Samples that were assayed for serum TNF levels were submitted to Dr. S. Harvey, Dept. Physiology, University of Alberta for determination of serum corticosterone levels from unstimulated and stimulated rats. Quantitation of serum corticosterone levels was done by using the method described (358). [1, 2, 6, 7-^{3}H]corticosterone (New England Nuclear, Boston, Mass, 1000 counts/min) was added to duplicate 75 uL portions of each plasma sample before extraction to determine efficiency of recovery of corticosterone. The samples were twice extracted with 1 mL redistilled ethanol and following centrifugation the combined supernatant fractions were evaporated under a stream of air in 15 mL conical glass centrifuge tubes at room temperature. The residue was concentrated in 200-300 uL redistilled ethanol and evaporated to dryness again. The residue was redissolved in 200 uL iso-octane:methanol: benzene (8:1:1 by volume), applied to a Sephadex LH-20 column (3.5 X 0.8 cm) and eluted with the same solvent. The elution profile of corticosterone on these columns was characterized using pure corticosterone, which did not cross-react in the assay (< 0.09 ng/mL), in charcoal-stripped serum (blank serum). The first 12 mL of the eluate were discarded and the next 12 mL collected. The elution patterns of pure [1α, 2α-^{3}H] 11-deoxycorticosterone (DOC; The Radiochemical Centre, Amersham, Bucks; 1.3-2.2 TBq/mmol), [1, 2, 6, 7-^{3}H]cortisol (The Radiochemical Centre, 3-3.9 TBq/mmol), [4-^{14}C]cholesterol (The Radiochemical Centre, >1.85 TBq/mmol), [1, 2-^{3}H]aldosterone (The Radiochemical Centre, >1.5-2.2 TBq/mmol), and [1, 2, 6, 7-^{3}H]progesterone (The Radiochemical Centre, 3-4.1 TBq/mmol) added to and extracted from blank plasma did

not significantly overlap that of corticosterone under these conditions. The corticosterone-containing eluate was then evaporated to dryness, redissolved in the eluting solvent, dried and dissolved in 500 uL PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, 0.1% gelatin, 0.1% sodium azide, pH 7.6). The tubes were then vortexed, sealed with Parafilm (Gallenkamp, London) and allowed to stand at room temperature for 48 hours. A portion (150 uL) of each sample was then counted in a Packard Tri-Carb scintillation counter (Packard Instruments, Illinois), to determine the efficiency of recovery, which ranged from 40-60%.

Antiserum to corticosterone was raised in New Zealand White rabbits immunized with 11 β, 21-dihydroxy-4-pregene-3, 20-dione hemissuccinate:bovine serum albumin conjugate (Steraloids Inc., Wilton) and at a final dilution used in the assay (1:18, 000) gave a bound:free ratio of 0.30. Cross-reaction of other steroid hormones with the antiserum, determined by titrating the antibody with doses of 10, 10^2, 10^3, 10^4, 10^5, and 10^6 pg of each steroid was minimal ($\leq$ 6.6%) except for DOC (100%) and progesterone (2.3%) could be removed from the plasma samples, together with plasma lipids, by chromatography with Sephadex LH-20.

Quadruplicate corticosterone standards (0.025, 0.05, 0.10, 0.20, 0.40, 0.60, 1.00, 2.00, 3.00, and 4.00 ng/tube) in 250 uL PBS or 250 uL of each sample were incubated with 500 uL anti-serum to corticosterone for 30 minutes at room temperature. Tritiated corticosterone 1000 counts/min) in 100 uL PBS was added to each of the tubes, which were vortexed and heated at 37°C for 1 hour and then incubated at 4°C for 24 hours. Dextran-coated charcoal (0 05% dextran sulphate; Pharmacia, CA.; 0.5% charcoal) was added to each tube in 200 uL ice-cold PBS, the tubes re-capped, inverted 10 times and incubated for 20 minutes at 4°C. Following centrifugation (10, 000 X g for 15 minutes at 4°C), the supernatant fractions were decanted into scintillation vials and counted in 7 mL scintillation fluid.

Concentrations of corticosterone in the samples were determined from a logit-log transformation of the standard curve. In 13 assays the minimum detectable dose (two standard deviations from maximal binding in the tubes containing no competing corticosterone) was 11.9 ± 1.0 pg/tube. The intra-assay coefficient of variation for the concentration (15.05 ± 0.31 (S.E.M.) ng/mL) of a pooled plasma sample assayed seven time in the same assay was 5.83% and the interassay coefficient of variation for its concentration (16.88 ± 0.52 ng/mL) in nine assays was 9.33%. The recovery of triplicate doses of unlabelled corticosterone (1.00 and 0.5 ng/mL) added to and extracted from blank plasma was 98 ± 1 and 106 ± 6% respectively. The recovery of higher doses of unlabelled corticosterone (4, 8 and 16 ng/mL) added to and extracted in triplicate from a pooled plasma (mean concentration 8.81 ± 0.24 ng/mL, $n = 6$) was 102 ± 2, 105 ± 4 and 105 ± 3%. The estimated concentration of corticosterone in ten plasma samples assayed with this antiserum and with a second antiserum also showed a good correlation (correlation coefficient, $r = 0.93$, $P < 0.001$). Dose-response inhibition curves parallel with those of the standard curve were also obtained with different volumes (150, 75, 50 and 25 uL) of a pooled plasma sample and its concentration was independent of plasma volume (21.17, 22.34, 21.92, and 23.31 ng/mL respectively).

TWO-COLOUR STAINING OF PERITONEAL CELLS AND FACS ANALYSIS

Aliquots of cells used for determination of released, cytosolic and membrane TNF levels were stained for leukocytes (OX-1; IgG_1; Cedarlane, Hornby, ON.)(359), peritoneal macrophages (OX-43; IgG_1)(360), Class I major histocompatibility complex (MHC)(OX-18; IgG_1; Cedarlane)(361), and Class II RT1.B (OX-6; IgG_1; Cedarlane)(361), and RT1.D (OX-17; IgG_1; Cedarlane)(361) MHC cell surface antigen expression. An isotype control antibody OX-21 (360) which recognizes mouse and human Factor I was included for determination of non-specific binding. OX-21 and OX-43 were kindly donated by Dr. A.A. Like (U. Mass.). OX-43 was purified by Protein A Sepharose (Pharmacia) and fluorescein isothiocyanate (FITC)-conjugated using FITC-celite (Calbiochem, San Diego, CA.). Unconjugated or FITC-conjugated OX-43 was used for macrophage staining while the other antibodies were unlabelled. The FITC- or phycoerythrin (PE)-conjugated second antibodies used were $F(ab_2)'$ fragments of goat anti-mouse Ig (heavy and light chain specific) absorbed against rat proteins (Cedarlane).

Peritoneal cells were washed twice in ice-cold PBS and the pellets were resuspended in PBS containing 10% (*v/v*) heat-inactivated goat serum (NGS; Cedarlane) and incubated for 30 minutes on ice to block non-specific antibody binding sites. Cells ($1\text{-}3 \times 10^5$ cells in 50 uL) were added to wells of a round bottom 96 well plate (Falcon; Becton Dickinson). All primary and secondary antibodies were prepared by appropriate dilution in PBS containing 10% (*v/v*) NGS.

Fifty microliters of the OX-43-FITC or unlabelled antibody was added to the appropriate wells, the cells resuspended and incubated on ice for 45-60 minutes. Cells were washed 3 times with ice cold PBS and once with ice cold PBS containing 10% (*v/v*) NGS and 50 uL of the subsequent unlabelled (or labelled antibody recognizing first determinant) antibody recognizing the second cell surface antigen was added. The plate was incubated for an additional 45-60 minutes on ice and in the dark. The cells were washed four times as above and the conjugated goat anti-mouse $F(ab_2)'$ antibody was added. Cells were incubated on ice and in the dark for 45 minutes and washed four times. After the last wash, the PBS was aspirated and the cells were fixed by adding 100 uL of 3% (*w/v*) formalin/well. The cell suspension was transferred to tubes and read on a FACSCAN cell analyzer (Becton-Dickinson).

INDIRECT DETECTION OF LIPOPOLYSACCHARIDE RECEPTORS ON PERITONEAL CELLS

Samples of cells used for determination of released, cytosolic, and membrane TNF levels were stained for lipopolysaccharide receptors (LPS-R) expressions using the methods described (362, 363) and modified for optimal detection in rat. One million cells/100 uL volume were plated into wells of a 96 well tissue culture plate to which fluorescein isothiocyanate (FITC)-conjugated LPS-*Salmonella minnesota* (FITC-LPS; Sigma, St. Louis, MO.) was added at a final concentration of 100 ug/mL in PBS was used to determine LPS-R or 10 ug/mL in PBS containing 10% (*v/v*) NGS to determine CD14 was added. The plate was incubated at 37°C for 60 minutes in a humidified incubator gassed with 95% air and 5% CO_2. The cells were then washed 4 times with ice-cold PBS and 50 uL of unlabelled OX-43 was added to the appropriate wells, the cells resuspended and incubated on ice in the dark for 45-60 minutes. The cells were washed four times with ice cold PBS and 50 uL of the PE-conjugated goat anti-mouse $F(ab_2)'$ antibody (Southern Biotechnology:

Birmingham, AL.) was added. The cells were incubated on ice and in the dark for an additional 45-60 minutes and washed four times. After the last wash, the PBS was aspirated and the cells were fixed by adding 100 uL of 3% (*w/v*) formalin/well. The cell suspensions were transferred to tubes and read on a FACSCAN cell analyzer (Becton-Dickinson).

STATISTICAL ANALYSIS

Results are expressed as means ± SE and statistical significance of the data were analyzed by the Student's *t* test for unpaired samples assuming that the data have a Gaussian distribution with equal standard deviations of the two populations compared, the Mann-Whitney test for unpaired samples assuming that the data do not have a Gaussian distribution, or the Fisher exact test using Instat V.2.01 software.

CHAPTER 3

TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE BB RATS IS DEFICIENT AND CAN BE CORRECTED BY TREATMENT WITH COMPLETE FREUND'S ADJUVANT[1]

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which the insulin-producing pancreatic islets are infiltrated primarily by autoreactive lymphocytes (insulitis), resulting in the selective destruction of the insulin-producing beta (β) cells by these cells (3-9).

Non-specific immunostimulation with adjuvants such as OK-432, Bovis Calmette-Guérin (BCG) and complete Freund's adjuvant (CFA) have been reported to prevent diabetes in the non-obese diabetic (NOD) mouse and BB rat (261, 262, 265, 266, 271, 272), and increased serum tumour necrosis factor (TNF) levels in the NOD mouse (269, 270). It is possible that adjuvant immunotherapy could prevent diabetes by increasing specific cytokine levels.

A role for cytokines in the prevention of IDDM in the BB rat and non-obese diabetic (NOD) mouse is suggested by the ability of chronic administration of the macrophage cytokine interleukin-1 (IL-1) (285, 286), the T lymphocyte cytokine IL-2 (280, 289), and the macrophage/T lymphocyte cytokine IL-10 (301), to significantly delay, or prevent the onset of diabetes. It has also been demonstrated in other studies that production of the macrophage cytokine, TNF-α was decreased in the NOD mouse (270, 328), and in the (NZB X NZW)F_1 mouse model of systemic lupus nephritis (364). Chronic administration of TNF-α prevented the development of these IDDM in the NOD mouse (270), and lupus nephritis in the (NZB X NZW)F_1 mouse (364, 365). Chronic administration of TNF-α or the T lymphocyte cytokine lymphotoxin (LT; TNF-β) were also capable of preventing IDDM in the BB rat and NOD mouse (329, 334, 335). These findings suggest that deficiencies in cytokines may participate in the pathogenesis of autoimmune disease.

Deficient production of cytokines including TNF-(α and β) may contribute to the onset and pathogenesis of autoimmune disease and this deficiency could be corrected by the administration of specific cytokines or non-specific immuno-adjuvants such as CFA. This hypothesis was tested by measuring TNF production by peritoneal macrophages from diabetes-prone (DP) BB rats after stimulation *in vivo* and after stimulation *in vitro*, and in DP rats protected against diabetes by CFA.

RESULTS

Peritoneal Cell TNF Production after Stimulation In Vivo

Diabetes-prone and DR rats were injected intravenously with saline, IFN-γ, LPS, or IFN-γ + LPS and 4 hours later, peritoneal cells were collected and cultured for 1.5 to 18 hours

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[1]A version of this chapter has been published by **Lapchak, P.H., L.J. Guilbert, and A. Rabinovitch**, *Clin. Immunol. Immunopathol. 65(2): 129-134, 1992.*

to measure TNF production (**FIGURE 3.1**). After 1.5 hours of culture, released and cytosol TNF levels were significantly lower in peritoneal cells from saline-treated and IFN-γ-treated DP rats compared to peritoneal cells from DR rats ($P \leq 0.01$ for released and for cytosol for saline, and $P \leq 0.05$ for released and for cytosol for IFN-γ). Released and cytosol TNF levels were lower, but not significantly at 3, 6, and 18 hours by peritoneal cells from saline-treated DP rats. In response to IFN-γ, released TNF by DP rat peritoneal cells at 3, 6 and 18 hours were lower, but not significant, however, cytosol TNF levels were significantly lower at 3 ($P \leq 0.01$) and 6 ($P \leq 0.05$) hours by DP rat compared to DR rat peritoneal cells. In response to LPS or IFN-γ + LPS, peritoneal cells from DP rats, had TNF (released and cytosol) levels that were lower, but not significantly compared to peritoneal cells from DR rats at all time points. In general, DP rat peritoneal cell TNF production (released and cytosol) is lower than DR rat peritoneal cells. Therefore, TNF production was compared at 1.5 hours of culture *in vitro* (**TABLE 3.1**). TNF levels (released, cytosol and total) were significantly lower by peritoneal cells from saline- and IFN-γ-treated DP rats compared to peritoneal cells from DR rats. In response to LPS, TNF levels were lower, but not significantly after treatment with LPS and were similar in response to IFN-γ + LPS.

Peritoneal Cell TNF Production after Stimulation In Vitro

Peritoneal cells were collected from untreated DP and DR rats and cultured *in vitro* for 3, 6 and 18 hours in medium alone, medium containing IFN-γ, LPS, or IFN-γ + LPS. There were no significant differences in TNF levels from DP rat peritoneal cells compared to DR peritoneal cells at 3, 6 and 18 hours of culture in medium alone (**FIGURE 3.2**). TNF levels were lower, but not significantly, by DP rat peritoneal cells at 6 hours but was significantly lower ($P \leq 0.05$) at 18 hours compared to DR rat peritoneal cells after culture in medium containing IFN-γ. TNF levels were significantly lower by DP rat peritoneal cells after 3 hours of culture in medium containing LPS ($P \leq 0.05$) compared to DR rat peritoneal cells and lower, but not significantly, at 6 and 18 hours. TNF levels were significantly lower by DP rat peritoneal cells compared to DR rat peritoneal cells after 3 hours ($P \leq 0.05$) and after 6 hours ($P \leq 0.05$) of culture in medium containing IFN-γ + LPS, and lower, but not significantly at 18 hours.

Effects of CFA Treatment of BB Rats on Peritoneal Cell TNF Production

Peritoneal cells were collected from CFA-treated DP and DR rats and cultured *in vitro* for 3, 6 and 18 hours in medium alone, medium containing IFN-γ, LPS, or IFN-γ + LPS (**FIGURE 3.3**). While TNF levels were greater by CFA-treated DR rat peritoneal cells compared to CFA-treated DP rat peritoneal cells in medium alone and medium containing IFN-γ, these differences were not significant. In response to LPS or IFN-γ + LPS, TNF levels by CFA-treated DP rat peritoneal cells were similar to TNF levels by CFA-treated DR peritoneal cells at all time points. **TABLE 3.2** shows that TNF levels by peritoneal cells from CFA-treated DP rats cultured in medium alone, medium containing IFN-γ, medium containing LPS, and medium containing IFN-γ + LPS are greater than levels by peritoneal cells from saline-treated DP rats, and are greater than those levels by peritoneal cells from saline-treated DR rats, and are similar to those levels by peritoneal cells from CFA-treated DR rats.

L929-8 Cell Sensitivity to Rat Peritoneal Cell TNF

To determine whether L929-8 cytolysis is rat TNF-mediated, a rabbit polyclonal antibody to murine TNF which cross-reacts with rat TNF was included in one experiment. As shown

in **FIGURE 3.4**, lysis of L929-8 cells by rat peritoneal cell TNF was inhibited by greater than 97% using an antibody titer between 10^3-80^3.

DISCUSSION

Recent evidence suggests that TNF may play a role in immunopathogenesis of autoimmune insulin-dependent diabetes mellitus. The results presented here demonstrate that unstimulated (PBS-injected) peritoneal cells and those peritoneal cells from DP rats stimulated *in vivo* with interferon-γ or LPS produce lower amounts of released and cytosolic TNF compared to peritoneal cells from diabetes-resistant (DR) rats. This suggests that both released and total (released + cytosol) TNF production by peritoneal cells from DP rats is deficient in unstimulated (naive) macrophages and after stimulation with IFN-γ or LPS. Deficient TNF production is evident early, at 1.5 hours of *ex vivo* culture and possibly reflects deficient macrophage TNF production *in vivo*.

The results presented here contrast the findings reported indicating that TNF production is upregulated in DP BB rats (336), however, macrophages studied by this group had been activated *in vivo* with *Corynebacterium parvum*, a potent and chronic macrophage activator 3 days prior to study *in vitro*.

These *in vivo* findings were confirmed when TNF production by peritoneal cells from DP rats was measured after culture *in vitro*. While TNF levels were similar by peritoneal cells from both DP and DR rats cultured in medium alone, TNF levels by peritoneal cells from DP rats were lower after culture in medium containing IFN-γ, in medium containing LPS, and in medium containing IFN-γ + LPS. In contrast, TNF levels by peritoneal cells from complete Freund's adjuvant (CFA)-treated DP rats were increased compared to TNF levels by peritoneal cells from PBS-treated DP rats. A single injection of CFA can prevent the development of insulitis and diabetes in BB rats (274), and NOD mice (273, 275). It has also been shown that CFA is capable of stimulating natural suppressor cell activity (273, 274), and suppressor macrophages (275). In this present study, the results suggest that one mechanism by which CFA may protect against diabetes development is by increasing macrophage TNF levels. The finding that CFA prevents diabetes by increasing peritoneal cell TNF levels is supported by other studies in which OK-432 administration to NOD mice, while protecting against diabetes development, increases serum TNF, but not IL-1, IL-2, or IFN-γ levels (269, 270) and the administration of LPSw which protected against diabetes in NOD mice and was equally as effective as administering TNF (264).

The results presented here are in agreement with other reports that autoimmune disease may be associated with deficient TNF production, and increasing TNF levels *in vivo* may be important in preventing disease. Tumour necrosis factor production was reported to be decreased in NOD mice (270, 328), and in (NZB X NZW)F_1 lupus nephritis mice (364), and that chronic administration of TNF prevented development of these autoimmune diseases (270, 328, 364). Similarly, a closely related molecule to TNF-α, lymphotoxin (LT) can also prevent diabetes in NOD mice after chronic administration (334). Furthermore, administration of IFN-γ, IL-1, or IL-2 were unable to prevent diabetes in NOD mice (270). Similarly, chronic administration of TNF or LT to DP rats also prevents diabetes (329, 335). Recently, it has been reported that administration of an antibody to TNF increased insulitis development (331), further supporting a protective role for TNF against diabetes development.

It remains to be determined how TNF acts to prevent autoimmune disease such as diabetes in NOD mice and BB rats. *In vivo* administration of TNF inhibits T and B lymphocyte proliferation in response to mitogens, inhibits NK cell and CTL-mediated cytotoxicity, and inhibits delayed type hypersensitivity responses (320), is a macrophage maturation/differentiation factor (322), activates macrophages (327), and participates in the development of the CD4 and CD8 T lymphocyte repertoire (322). Therefore, TNF may act directly to inhibit autoreactive T lymphocyte-mediated killing, may participate in returning to normal, T lymphocyte ontogeny in DP rats, or act to initiate maturation/differentiation and activation of DP rat macrophages to the suppressor macrophage state. Tumour necrosis factor may act directly to prevent diabetes in BB rats by inhibiting the actions of autoreactive islet cell-specific T lymphocytes, or it may inhibit these cells through the generation of suppressor macrophages. Lastly, a deficient TNF production by macrophages may result in cascade of cytokine deficiencies, since TNF, but not IL-1 (α and β), IL-6, or GM-CSF, is important inducing *de novo* production of IL-10 mRNA and secretion (366), which in turn can act on inhibiting T lymphocyte activation, proliferation and secretion of IL-2 and IFN-γ (366).

SUMMARY

Peritoneal cells from DP rats produce deficient levels of TNF after stimulation *in vivo* or after stimulation *in vitro*. Deficient TNF production by peritoneal cells from DP rats can be upregulated by immunotherapy with complete Freund's adjuvant suggesting that TNF is important is preventing diabetes in the BB rat. The administration of antibodies to TNF will clarify whether CFA prevention of diabetes in BB rats is mediated through increasing TNF production.

TABLE 3.1

TNF PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS

BB rat group	Treatment *in vivo*	TNF (pg/10^6 cells/1.5 hr)		
		Medium	Cytosol	Total
DR	Saline	44.5 ± 7.2	18.0 ± 1.5	62.5 ± 8.3
DP	Saline	20.4 ± 3.4**	5.2 ± 0.4**	25.5 ± 3.5**
DR	IFN-γ	178.5 ± 42.6	42.1 ± 9.9	220.6 ± 49.2
DP	IFN-γ	36.7 ± 4.4*	13.6 ± 3.0*	50.3 ± 6.4*
DR	LPS	113.6 ± 38.9	36.9 ± 9.9	150.5 ± 47.4
DP	LPS	71.2 ± 14.0	16.8 ± 4.6	88.0 ± 16.6
DR	IFN-γ/LPS	130.7 ± 48.2	14.2 ± 5.6	144.9 ± 50.2
DP	IFN-γ/LPS	120.9 ± 42.6	19.6 ± 3.5	140.6 ± 45.8

Note: Values are Means ± S.E. for TNF levels in cell incubation medium, cytosol, and total (medium + cytosol) of peritoneal cells from 2 matched experiments for 6 DP and 6 DR rats injected with saline, IFN-γ, LPS, or IFN-γ followed with LPS as described in *Materials and Methods.* Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

*$P \leq 0.05$, ** $P \leq 0.01$ for DP *vs.* DR rats.

TABLE 3.2

EFFECTS OF TREATMENT OF BB RATS WITH COMPLETE FREUND'S ADJUVANT (CFA) ON TNF PRODUCTION BY PERITONEAL CELLS

BB rat group	Treatment *in vivo*	TNF (pg/10^6 cells/6 hr) in culture containing			
		Medium	IFN-γ	LPS	IFN-γ/LPS
DP	Saline	7.6 ± 2.6	17.1 ± 9.0	33.2 ± 11.4	30.9 ± 10.5
DP	CFA	18.2 ± 11.8	31.8 ± 11.4	84.8 ± 25.6	68.3 ± 7.7*
DR	Saline	9.0 ± 2.3	27.0 ± 4.0	45.1 ± 13.2	47.5 ± 10.1
DR	CFA	42.2 ± 33.5	66.1 ± 56.5	100.6 ± 45.3	49.7 ± 21.1

Note: Diabetes-prone (DP) and diabetes-resistant (DR) BB rats (ages 22-28 days) were injected once with saline or complete Freund's adjuvant (CFA), then 10-14 days later peritoneal cells were collected and incubated for 6 hr in control medium, and medium containing interferon-γ (IFN-γ, 100 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL) or IFN-γ + LPS. Values are Means ± S.E. for TNF levels in cell incubation medium are shown for 4 DP and 4 DR rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

* $P \leq 0.05$ for DP-CFA *vs.* DP-saline.

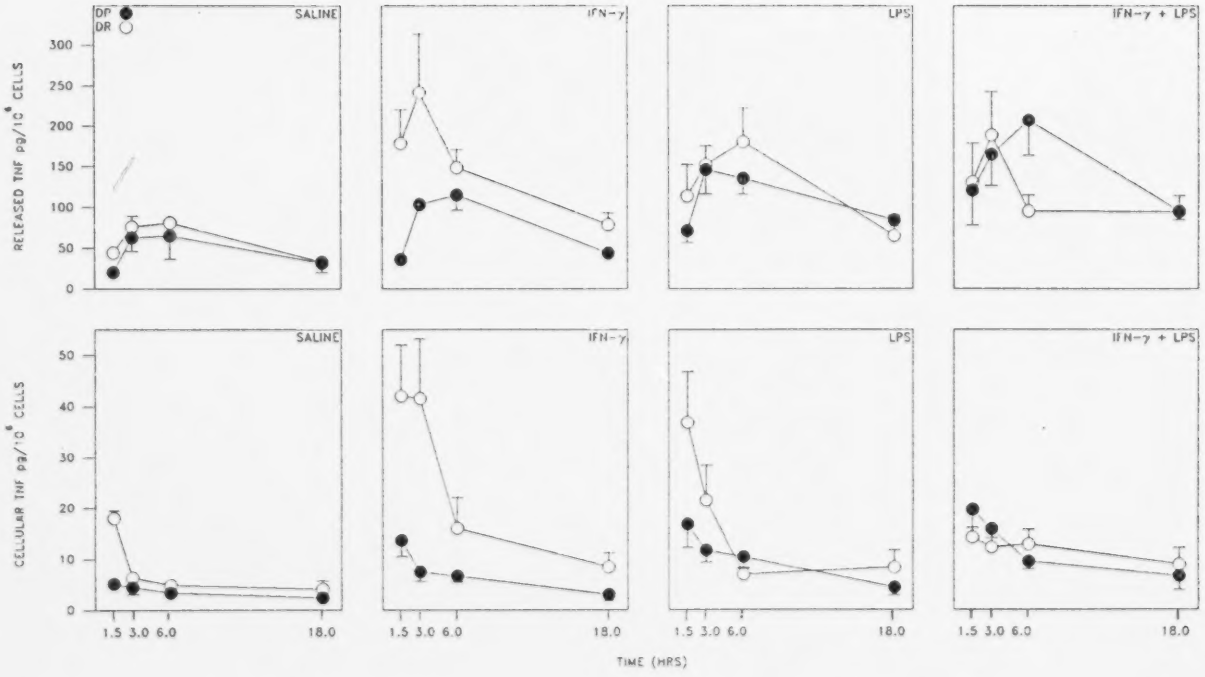

FIGURE 3.1

TNF Production by Peritoneal Cells From Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vivo*. DR and DP BB rats (ages 35-45 days) were injected intravenously with saline, interferon-γ (IFN-γ, 10^5 U), lipopolysaccharide (LPS, 500 ug) or IFN-γ + LPS as described in *Materials and Methods*. Four hours later, peritoneal cells were collected and incubated for 1.5, 3, 6, and 18 hours in complete medium. TNF levels in cell incubation medium and in cell cytosol (Mean ± S.E.) are shown for 6 animals from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

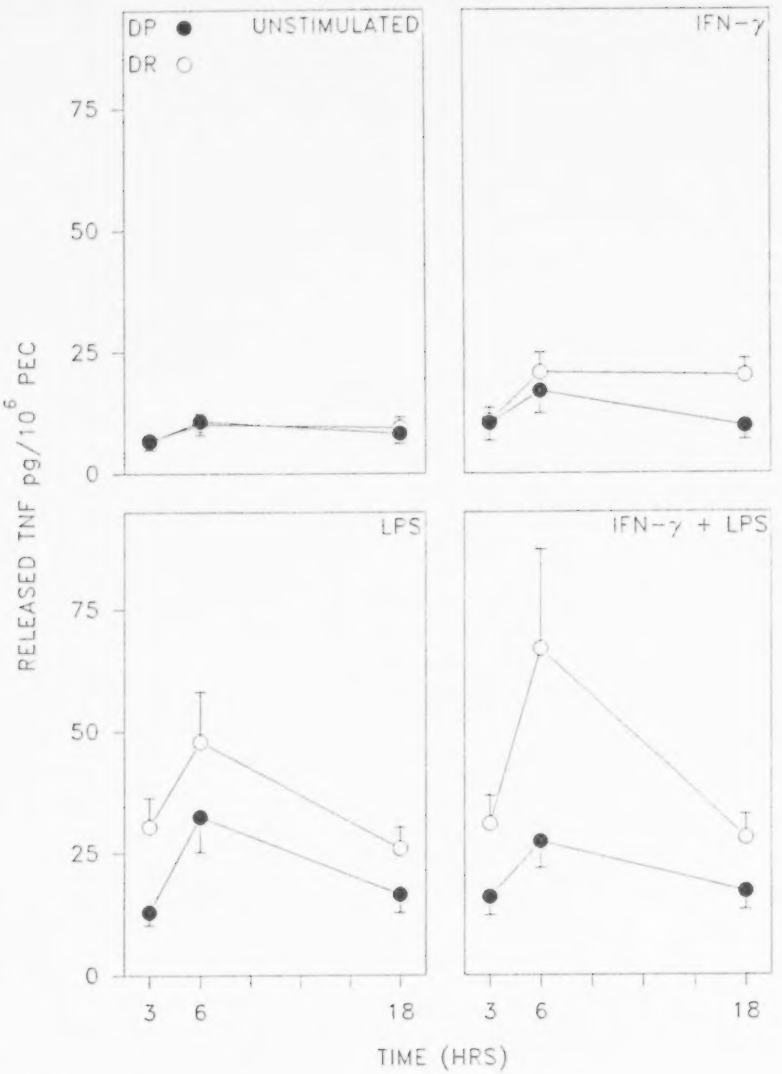

FIGURE 3.2

TNF Production by Peritoneal Cells From Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vitro*. DR and DP BB rats (ages 23-41 days) were collected and cultured for 3, 6, and 18 hours in complete medium alone, and medium containing interferon-γ (IFN-γ, 10^2 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL) or IFN-γ + LPS as described in *Materials and Methods*. TNF levels (Mean ± S.E.) are shown for 7 DR and 7 DP rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

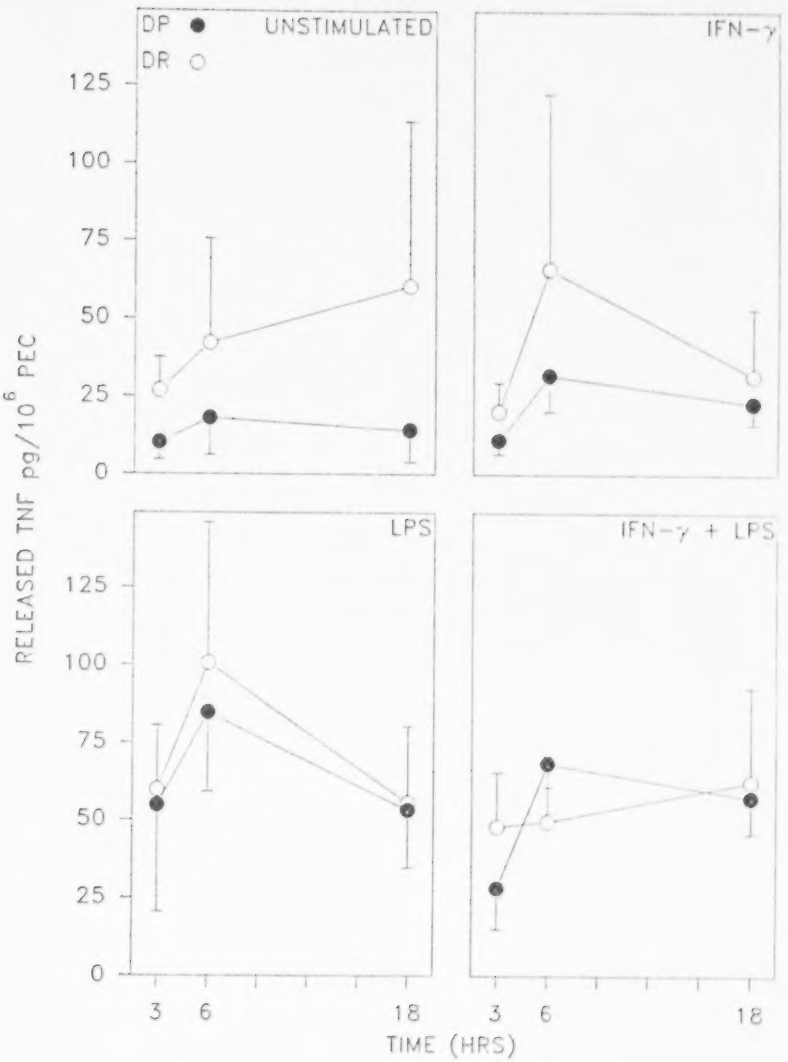

FIGURE 3.3
TNF Production by Peritoneal Cells From Complete Freund's Adjuvant (CFA)-Treated Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vitro*. DR and DP BB rats (ages 28-41 days) were collected and cultured for 3, 6, and 18 hours in complete medium alone, and medium containing interferon-γ (IFN-γ, 10^2 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL) or IFN-γ + LPS as described in *Materials and Methods*. TNF levels (Mean ± S.E.) are shown for 3 DR and 3 DP rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

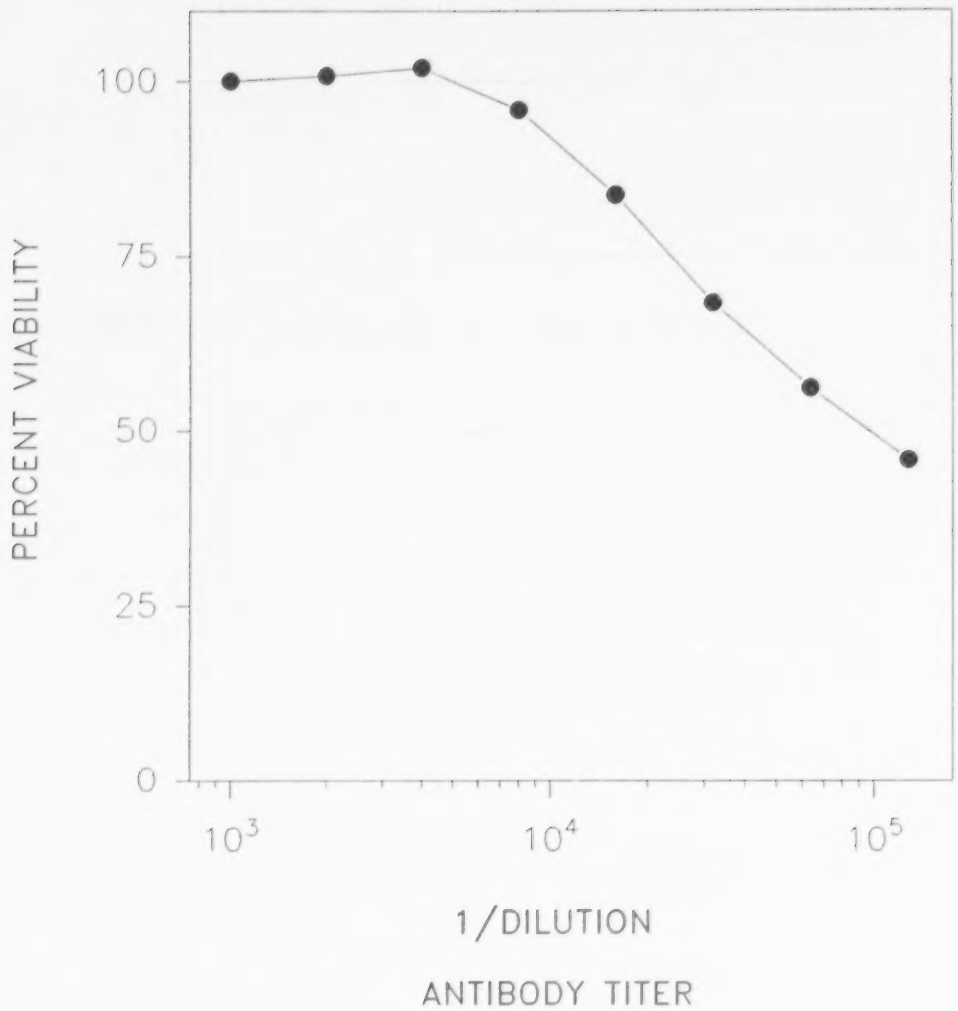

FIGURE 3.4
Neutralization of Rat Peritoneal Cell TNF-Mediated Cytotoxicity of L929-8 Cells by a Rabbit Polyclonal Antibody to Murine TNF. Rat peritoneal cell TNF was pre-cultured with dilutions of a polyclonal antibody to murine TNF and the mixture then added to the L929-8 TNF bioassay as described in *Materials and Methods*. Inhibition of TNF-mediated killing of L929-8 cells were compared to L929-8 cells cultured alone or containing rat peritoneal cell TNF. Results from one experiment are expressed as cell viability *vs.* antibody titer.

CHAPTER 4

EXCESSIVE TUMOUR NECROSIS FACTOR PRODUCTION BY SPLEEN CELLS FROM DIABETES-PRONE BB RATS IS DECREASED BY TREATMENT *IN VIVO* WITH COMPLETE FREUND'S ADJUVANT

INTRODUCTION

Insulin-dependent diabetes mellitus is an autoimmune disease manifested predominantly by activated autoreactive T lymphocytes that destroy the insulin-producing cells in the islets of Langerhan (5-9). Like macrophages, tumour necrosis factor alpha (TNF-α) is also produced by T and B lymphocytes, and natural killer (NK), however, these cells predominantly produce lymphotoxin (LT, also called TNF-β)(310, 318, 319).

Studies *in vitro* have shown that TNF-α and LT can act in synergy with other cytokines such as IL-1, or IFN-γ to be directly cytotoxic to islets (147-149, 151, 152). This is in accord with the presence of TNF- or IL-1-mRNA$^+$ lymphocytes found in intra-islet and peri-islet lesions in the BB rat (159), in intra-islet lesions in NOD mice (367), and TNF-β in isolated lymphocytes from islets of female NOD mice 5-16 weeks old and male NOD mice 7-24 weeks old (368).

A role for TNF-α or LT in the pathogenesis of human autoimmune disease is supported by the finding of excessive TNF-α and LT production from T lymphocytes isolated or cloned from thyroid infiltrates in Hashimoto's thyroiditis, to a lesser extent in Grave's disease (369, 370), and from synovial membranes in rheumatoid arthritis (370). In animal models of autoimmune disease, both TNF-α and LT mRNA were found in CD4$^+$ and CD8$^+$ lymphocytes from *lpr* mice which develop an autoimmune syndrome resembling systemic lupus erythematosus and rheumatoid arthritis (371), CD4$^+$ lymphocytes from C3H-*lpr/lpr* and C3H-*gld/gld* mice over-produce TNF (372), and myelin-basic protein-specific T lymphocyte clones from a mouse model of multiple sclerosis, experimental allergic encephalomyelitis, also over-expressed TNF/LT mRNA and over-produced TNF/LT protein upon stimulation (373).

Non-specific immunostimulation with complete Freund's adjuvant (CFA) reduces *in vitro* proliferative responses of lymphocytes from NOD mice to alloantigen and to mitogens (272, 275) while increasing IL-4 levels and reducing the IFN-γ levels (278). Together, this modulation of cytokine production by CFA may act to suppress lymphocyte TNF production.

Over-production of cytokines including TNF-α and LT by autoreactive lymphocytes may contribute to the specific destruction of insulin-producing beta cells in the islets, and this over-production may be down-regulated by administration of specific cytokines or non-specific immuno-adjuvants such as CFA. This hypothesis was tested by measuring TNF production by spleen cells from diabetes-prone (DP) BB rats after stimulation *in vivo* and after stimulation *in vitro*, and in DP rats protected against diabetes by CFA.

RESULTS

Spleen Cell TNF Production after Stimulation In Vivo

In a preliminary experiment, DP and DR rats were injected intravenously with saline, IFN-γ, LPS, or IFN-γ + LPS and 4 hours later, spleen cells were collected and cultured for 3, 6, and 18 hours to measure TNF production (**FIGURE 4.1**). Released TNF levels by saline-treated and IFN-γ-treated DP and DR rat spleen cells were similar at all three time points. Released TNF levels by spleen cells from LPS-treated DP rats were lower, but not significantly, at 3, 6, and 18 hours compared to spleen cells from LPS-treated DP rats compared to LPS-treated DR rats. Released TNF by spleen cells cultured for 3 hours from IFN-γ + LPS-injected DP rats were significantly greater than by spleen cells from IFN-γ + LPS-treated DR rats ($P \leq 0.05$), and were greater, but not significantly at 6 hours of culture, and were similar at 18 hours of culture.

In a second experiment, DP and DR rats were treated as above, and spleen cells were collected and cultured for 1.5, 3, 6, and 18 hours to measure released and cytosolic TNF levels (**FIGURE 4.2**). Released and cytosolic TNF levels were similar at all time points by spleen cells from saline-treated DP and DR rats. Released and cytosolic TNF levels by spleen cells from IFN-γ-treated DP rats were not significantly different at any time point compared to spleen cells from IFN-γ-treated DR rats. Released TNF levels by spleen cells from LPS-treated DP rats were similar to spleen cells from LPS-treated DR rat spleen cells at 1.5 and 3 hours. Cytosolic TNF levels by LPS-treated DP spleen cells were greater, but not significantly, compared to LPS-treated DR spleen cells, at 1.5, 3, and 6 hours. Released TNF levels by spleen cells from LPS-treated DP rats were significantly greater than by spleen cells from LPS-treated DR rats at 6 ($P \leq 0.05$) and 18 ($P \leq 0.05$), hours while cytosolic TNF levels from spleen cells from DP rats were lower compared to spleen cells from DR rats, but not significantly, at 18 hours. TNF levels by spleen cells from IFN-γ + LPS-treated DP rats were similar to spleen cells from IFN-γ + LPS-treated DR rats after 1.5, 3, and 6 hours of culture, while cytosolic TNF levels were greater, but not significantly, at the same time points. At 18 hours of culture, released TNF by spleen cells from IFN-γ + LPS-treated DP rats were significantly greater compared to spleen cells from DR rats ($P \leq 0.05$).

In general, DP rat spleen cell TNF production (released and cytosolic) is greater than by spleen cells from DR rats. TNF production was compared after 18 hours of incubation *ex vivo* (**TABLE 4.1**). TNF levels (released, cytosolic and total) were similar for saline- or IFN-γ-treated DP rat spleen cells compared to spleen cells from DR rats. TNF levels (released, and total) were significantly greater by spleen cells from LPS- and IFN-γ + LPS-treated DP rats compared to spleen cells from DR rats ($P < 0.05$ for released and for total).

Spleen Cell TNF Production after Stimulation In Vitro

Spleen cells from untreated DP and DR rats were cultured *in vitro* for 3, 6 and 18 hours in medium alone, in medium containing IFN-γ, LPS, or IFN-γ + LPS. There were no significant differences in released TNF levels by unstimulated DP rat spleen cells compared to DR spleen cells at after culture for 3, 6 and 18 hours (**FIGURE 4.3**). DP rat spleen cell TNF levels were lower but not significantly, compared to DR rat spleen cells at all time points after culture in medium containing IFN-γ. DP rat spleen cell TNF levels were greater, but not significantly, compared to DR rat spleen cells at all time points after culture in medium containing LPS. DP rat spleen cell TNF levels were lower at 3 hours,

greater at 6 hours and similar at 18 hours, but not significantly, compared to DR rat spleen cells after culture in medium containing IFN-γ + LPS.

Effects of CFA Treatment of BB Rats on Spleen Cell TNF Production
Spleen cells from CFA-treated DP and DR rats were cultured *in vitro* for 3, 6 and 18 hours in medium alone, in medium containing IFN-γ, LPS, or IFN-γ + LPS (**FIGURE 4.4**). Released TNF by spleen cells from CFA-treated DP rats were similar and not significantly different to spleen cells from CFA-treated DR rats at all time points and in all culture conditions. **TABLE 4.2** shows that peak levels of released TNF by spleen cells from CFA-treated DP rats cultured in medium alone, in medium containing IFN-γ, in medium containing LPS, and in medium containing IFN-γ + LPS are similar to those levels released by spleen cells from saline-treated DP and DR rats and CFA-treated DR rats.

DISCUSSION

Tumour necrosis factors may play a role in the immunopathogenesis of autoimmune disease and have been demonstrated *in vitro* to synergize with other cytokines to mediate specific islet cell killing. The results presented in a preliminary experiment demonstrate that spleen cells from DP rats stimulated *in vivo* with IFN-γ + LPS produced greater amounts of TNF compared to spleen cells from DR rats. This preliminary finding was confirmed in a second experiment. Here, DP spleen cell TNF levels (released and cytosolic) were greater at later time points in response to IFN-γ, LPS, and IFN-γ + LPS, and was greatest at 18 hours of *ex vivo* culture. These results are supported by other reports describing over-production of TNF by T lymphocytes or T lymphocyte clones from a variety of autoimmune diseases (368-373). This finding suggests that excessive production of TNF by spleen cells, if confirmed in the islets, may participate in the onset of diabetes by directly killing insulin-producing β cells.

TNF production by spleen cells from DP rats was measured after stimulation *in vitro* in an attempt to confirm the *in vivo* findings. No significant differences in TNF production could be seen between spleen cells from DP and DR rats. In addition, *in vitro* stimulation of TNF production by spleen cells from CFA-treated DP rats were similar to spleen cells from PBS-treated DP rats, and this contrasts the potentiating effect seen with spleen cells from CFA-treated DR rats compared to PBS-treated DR rats. These results suggest that CFA may regulate DP rat spleen cell TNF production directly or indirectly through other cells resident in the spleen such as macrophages. Clearly, it would be of great interest that CFA could act directly on spleen cells by down-regulating LT through up-regulation of TNF-α.

The dichotomy in TNF production after stimulation *in vivo* and *in vitro* may be a consequence of the microenvironments. Within the spleen, there are T lymphocyte subsets, B lymphocytes, dendritic cells, macrophages, and NK cells. As a consequence of this microenvironment, cells are in close proximity to each other and are more likely influenced by the action of different cytokines, both stimulatory and suppressive and cell-cell interactions. In contrast, cells stimulated *in vitro* may not be in close proximity and have fewer cell-cell contacts, thereby reducing the potential for stimulation through these contacts or through the paracrine effects of cytokines.

Tumour necrosis factor from these *in vivo* and *in vitro* experiments were measured by the L929-8 bioassay. There are no polyclonal or monoclonal antibodies available to

discriminate between different species of rat TNF. As a result, it was impossible to distinguish whether DP rat spleen cells are over-producing LT-α/β or TNF-α.

Complete Freund's adjuvant-mediated protection of diabetes in NOD mice and BB rats is partly attributed to the increase in numbers of natural suppressor cells in the spleen resulting in a decrease in proliferation to allo-antigen or mitogen stimulation (272, 274, 275), a state of dormancy of the autoreactive T lymphocytes (277), and an increase in the numbers of IL-4-producing cells (278). These actions may be the result of increased TNF production by macrophages after CFA-treatment (Chapter 3).

Although it is likely that spleen cells produce LT predominantly, they could also produce TNF-α to a lesser extent (310, 318, 319) and that "appropriate" amounts of TNF may be protective (374). Both TNF-α and LT can mediate their effects through the tumour necrosis factor receptors (TNF-R) of 55 kDa and 75 kDa (375). More recently, a new receptor has been described, and is specific for LT-α:LT-β heterodimers and may serve to function as positive or negative regulator in inflammatory and immune responses (376). However, responses specific to this receptor have yet to be described.

While LT has been shown to be an effector molecule, it is not clear whether it is involved in the production of "suppressor" cytokines such as IL-4 or IL-10. It has been demonstrated that macrophage derived TNF-α is essential in inducing macrophage derived IL-10 (366). One action that CFA may mediate in the spleen of DP rats could be to down-regulate or suppress T lymphocyte LT production via induction of *de novo* IL-10 in spleen macrophages or T_{h2} lymphocytes through the induction of TNF-α (366). What may be observed in the spleens of DP rats is a dysregulation of TNF, namely LT as a result of deficient IL-4 or IL-10 production.

Clarification of the species of TNF stimulated *in vivo* will be required. Purification of spleen cell subsets, namely helper and cytotoxic T lymphocytes, B lymphocytes, NK cells and macrophages, will facilitate the identity of TNF species. Furthermore, using specific probes for TNF-α, LT, and LT-β will allow identification of *de novo* induction of TNFs through the actions of CFA in spleen cells of DP rats.

SUMMARY

Spleen cells from DP rats produce excessive amounts of TNF compared to DR spleen cells after stimulation *in vivo* and was corrected by CFA. However, *in vitro* stimulation of DP or CFA-treated DP rat spleen cells results in similar levels of TNF production, whereas it is increased by spleen cells from CFA-treated DR rats compared to PBS-treated DR rats. This suggests that *in vivo* there may be induction of suppressive cytokines in spleen cells from CFA-treated DP rats which suppresses TNF production. Additional studies using purified spleen cell subsets from *in vivo*-stimulated DP rats are necessary to determine the cell source(s) of spleen cell TNF over-production and to determine, albeit indirectly, which species of TNF is predominant. Additionally, experiments could be designed to demonstrate which TNF species, that is, TNF-α, LT, or LT-β may be important intrasplenically to regulate effector and suppressor cytokine levels.

TABLE 4.1

TNF PRODUCTION BY SPLEEN CELLS OF DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS

BB rat group	Treatment *in vivo*	TNF (pg/10^6 cells/18 hr)		
		Medium	Cytosol	Total
DR	Saline	4.2 ± 1.9	1.3 ± 0.5	5.4 ± 1.9
DP	Saline	4.0 ± 1.2	0.3 ± 0.1	4.4 ± 1.1
DR	IFN-γ	2.5 ± 0.1	1.0 ± 0.4	3.4 ± 0.5
DP	IFN-γ	4.6 ± 1.6	0.4 ± 0.1	4.9 ± 1.6
DR	LPS	5.9 ± 1.3	2.0 ± 0.9	7.9 ± 2.1
DP	LPS	18.5 ± 4.1*	0.7 ± 0.2	19.2 ± 4.3*
DR	IFN-γ/LPS	8.6 ± 1.2	2.4 ± 1.0	11.0 ± 1.7
DP	IFN-γ/LPS	27.9 ± 5.6*	1.3 ± 0.3	29.0 ± 5.6*

Note: Values are Means ± S.E. for TNF levels in cell incubation medium, cytosol, and total (medium plus cytosol) by spleen cells from 2 matched experiments for 6 DP and 6 DR rats injected with saline, IFN-γ, LPS, or IFN-γ followed by LPS as described in *Materials and Methods*. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

*$P < 0.05$ for DP rats *vs.* DR rats.

TABLE 4.2

EFFECTS OF TREATMENT ON BB RATS WITH COMPLETE FREUND'S ADJUVANT (CFA) ON TNF PRODUCTION BY SPLEEN CELLS

BB rat group	Treatment *in vivo*	TNF (pg/10^6 cells/18 hr) in culture containing			
		Medium	IFN-γ	LPS	IFN-γ/LPS
DP	Saline	0.3 ± 0.1	1.3 ± 0.4	21.6 ± 7.7	17.8 ± 8.8
DP	CFA	1.2 ± 0.4	2.3 ± 0.9	20.7 ± 4.4	22.8 ± 6.2
DR	Saline	1.0 ± 0.5	5.3 ± 2.4	17.0 ± 3.1	24.2 ± 10.0
DR	CFA	1.3 ± 1.0	2.3 ± 1.1	10.5 ± 5.4	57.4 ± 42.4

Note: Diabetes-prone (DP) and diabetes-resistant (DR) BB rats (ages 22-28 days) were injected once with saline or complete Freund's adjuvant (CFA), then 10-14 days later spleen cells were collected and incubated for 18 hours in control medium,in medium containing interferon-γ (IFN-γ, 100 U/mL), in medium containing lipopolysaccharide (LPS, 0.5 ug/mL) or in medium containing IFN-γ + LPS. Values are Means ± S.E. for TNF levels in medium for 4 DP and 4 DR rats from 2 matched experiments.

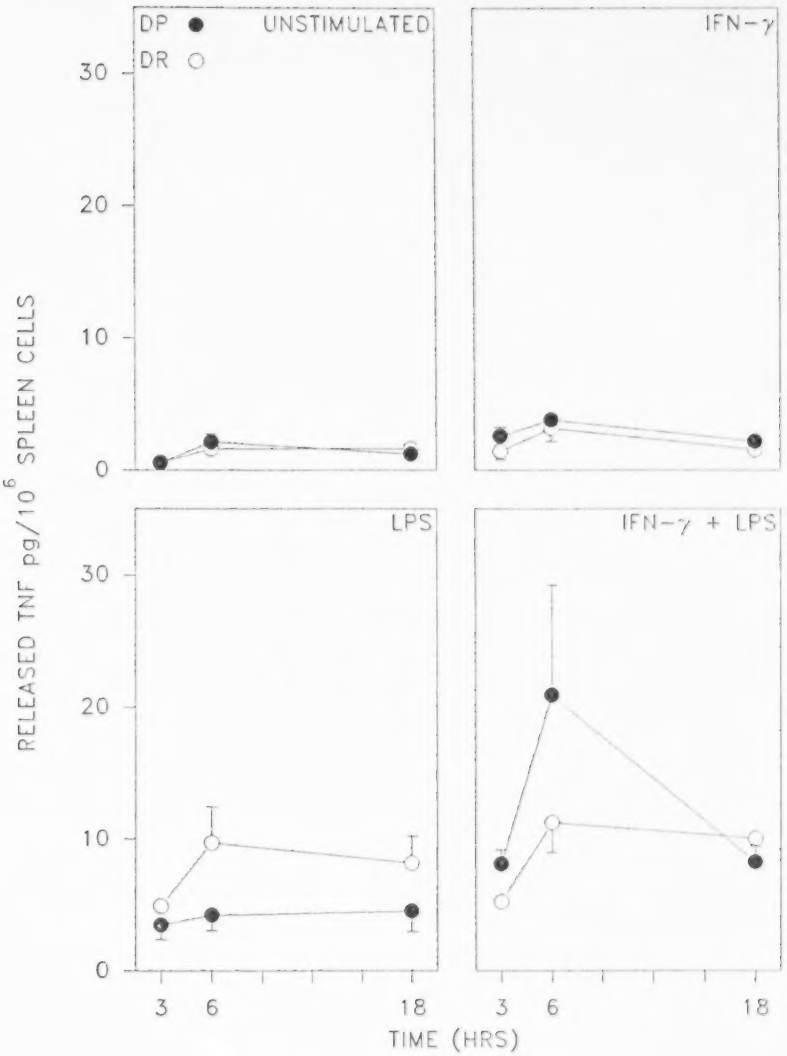

FIGURE 4.1
TNF Production by Spleen Cells From Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vivo*. DR and DP BB rats (ages 24-45 days) were injected intravenously with saline, interferon-γ (IFN-γ, 10^5 U), lipopolysaccharide (LPS, 500 ug) or IFN-γ + LPS as described in *Materials and Methods*. Four hours later, spleen cells were collected and incubated for 3, 6, and 18 hours in complete medium. TNF levels in incubation media (Mean ± S.E.) are shown for 6 DP and 6 DR rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

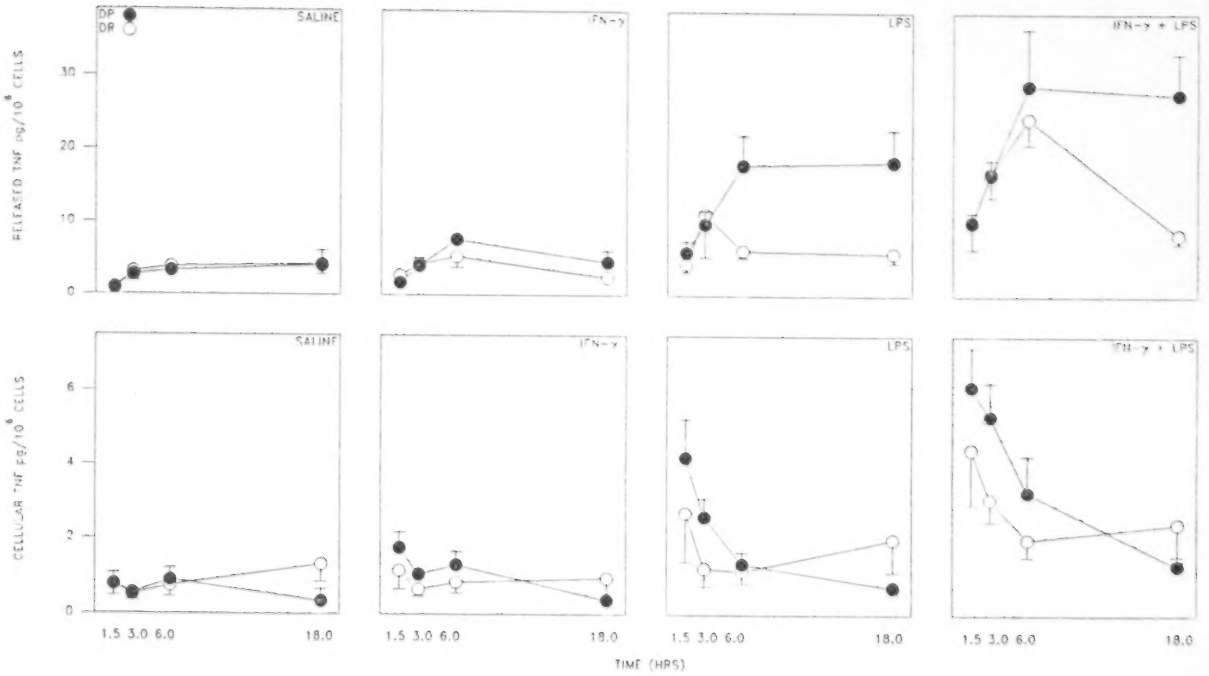

FIGURE 4.2

TNF Production by Spleen Cells From Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vivo*. DR and DP BB rats (ages 35-45 days) were injected intravenously with saline, interferon-γ (IFN-γ, 10^5 U), lipopolysaccharide (LPS, 500 ug) or IFN-γ + LPS as described in *Materials and Methods*. Four hours later, spleen cells were collected and incubated for 1.5, 3, 6, and 18 hours in complete medium. TNF levels in cell incubation media or cell cytosol (Mean ± S.E.) are shown for 6 DP and 6 DR rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

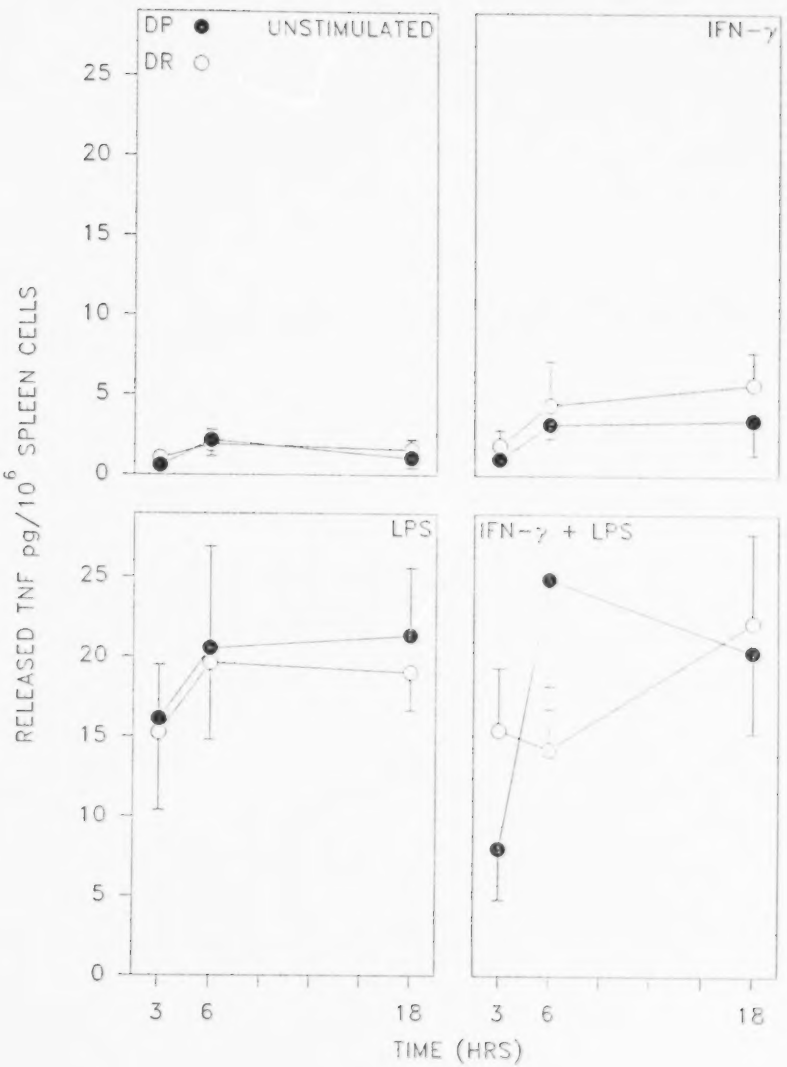

FIGURE 4.3
TNF Production by Spleen Cells From Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vitro*. Spleen cells from DR and DP BB rats (ages 23-41 days) were collected and cultured for 3, 6, and 18 hours in complete medium alone, and medium containing interferon-γ (IFN-γ, 10^2 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL) or IFN-γ + LPS as described in *Materials and Methods*. TNF levels (Mean ± S.E.) are shown for 6 DP and 6 DR rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

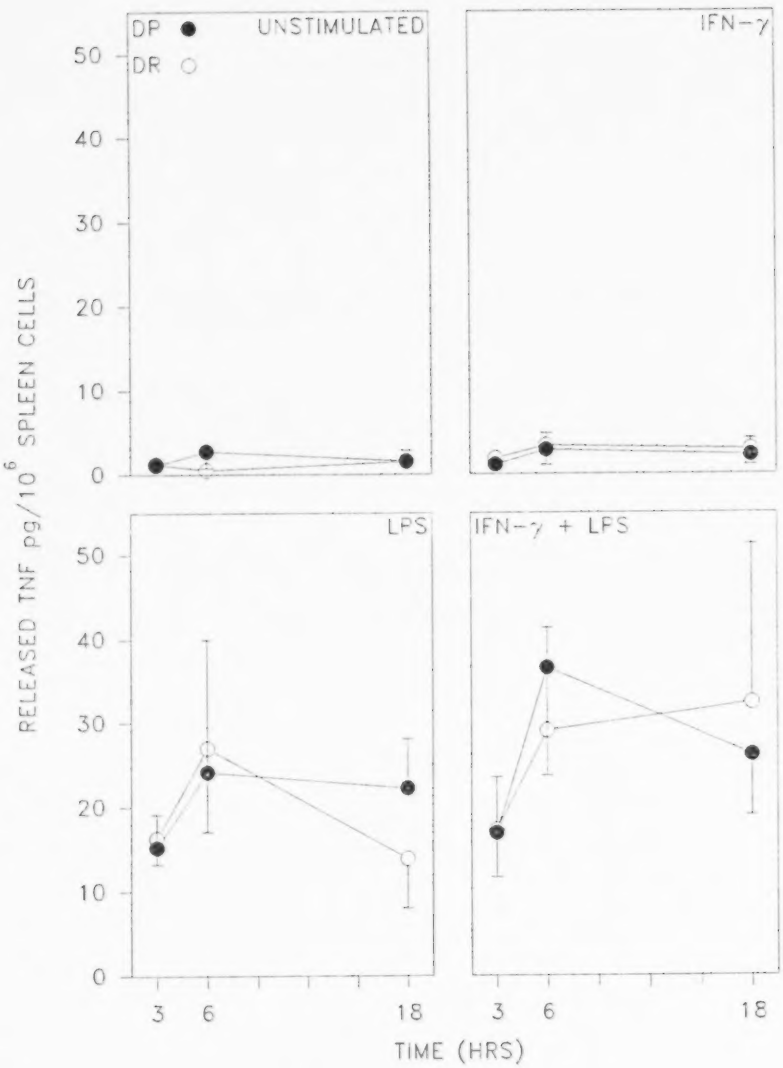

FIGURE 4.4

TNF Production by Spleen Cells From Complete Freund's Adjuvant (CFA)-Treated Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vitro*. Spleen cells from DR and DP BB rats (ages 28-41 days) were collected and cultured for 3, 6, and 18 hours in complete medium alone, and medium containing interferon-γ (IFN-γ, 10^2 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL) or IFN-γ + LPS as described in *Materials and Methods*. TNF levels (Mean ± S.E.) are shown for 3 DP and 3 DR rats from 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

CHAPTER 5

DYSREGULATION OF TUMOUR NECROSIS FACTOR PRODUCTION IS TISSUE-SPECIFIC AND CORRECTED AFTER TREATMENT *IN VIVO* WITH COMPLETE FREUND'S ADJUVANT

INTRODUCTION

Tumour necrosis factor (TNF) is produced by a large number of lymphoid cells including macrophages and monocytes, T and B lymphocytes, natural killer (NK) cells and thymocytes (310, 318, 319), is involved in regulating cell-mediated immunity (320), is a macrophage maturation/differentiation factor (321), and is involved in the development of the T lymphocyte repertoire (322, 323).

In chapters 3 and 4, it was demonstrated that TNF production is dysregulated in peritoneal cells and spleen cells from diabetes-prone (DP) BB rats, and that this dysregulation can be corrected by adjuvant immunotherapy with complete Freund's adjuvant (CFA).

BB rats are severely T lymphopenic (15, 39-44), and have apparently no $RT6^+$ T lymphocytes (47, 48) thought to arise from defective thymic development (50) post-thymic maturation (51), or through inefficient CD4:CD45RA/RB co-receptor signalling through aberrant TNF production (63, 69), resulting in the generation of autoreactive T lymphocytes (64). Furthermore, DP rats contain reduced numbers of $CD4^+$ T lymphocytes and virtually no $CD8^+$ T lymphocytes (15, 43, 44).

Tumour necrosis factor is important in regulating cell-mediated immunity without affecting humoral immunity (320), is a growth-promoting factor for thymocytes (377), is involved in the expression of CD8 on thymic pre-T lymphocytes (378, 379), and for increasing expression of the interleukin-2 receptor-alpha and-beta chains (380).

Defective TNF production in the DP rat thymus may result in abnormal T lymphocyte ontogeny which gives rise to T lymphopenia characterized by decreased $CD4^+$ and absent $CD8^+$ T lymphocytes. Furthermore, TNF over-production in the spleen may impede post-thymic maturation and differentiation by lymphocytes. This hypothesis was tested by measuring cytosolic TNF levels in freshly isolated thymocytes, and in peritoneal and spleen cells from untreated and from CFA-protected DP rats, and measuring released and cytosolic TNF levels in thymocytes, peritoneal and spleen cells from untreated and CFA-treated DP rats cultured for 6 hours after stimulation *in vitro*.

RESULTS

Tissue-Specific Dysregulation of Tumour Necrosis Factor in Freshly Isolated Peritoneal Cells After Culture In Vitro

Tumour necrosis factor production was measured in freshly isolated peritoneal cells, spleen cells and thymocytes from saline-treated and from CFA-treated DP and DR rats, or after 6 hours of *in vitro* culture. **Table 5.1** shows cytosolic TNF levels from freshly isolated peritoneal cells, spleen cells and thymocytes. TNF levels by peritoneal cells and spleen cells from saline- and CFA-treated DP rats are similar, while thymocyte cytosolic TNF levels from CFA-treated DP rats are significantly greater than by saline-treated DP and CFA-treated DR rats. Cytosolic TNF levels by peritoneal cells from CFA-treated DR rats are

significantly greater compared to cytosolic TNF levels by peritoneal cells from saline-treated DR rats, and from CFA-treated DP rats. Cytosolic TNF levels by spleen cells from CFA-treated DR rats is significantly greater than from saline-treated DR rats, but not significantly different from spleen cells from saline- or CFA-treated DP rats. No significant differences are seen in cytosolic TNF by thymocytes from saline- or CFA-treated DR rats.

Effect of Complete Freund's Adjuvant Treatment of Diabetes-Prone BB Rats on Tumour Necrosis Factor Production by Peritoneal Cells After Culture In Vitro

Peritoneal cells from saline- and CFA-treated DP and DR rats were cultured *in vitro* for 6 hours in medium alone, in medium containing IFN-γ, or in medium containing LPS after which time released and cellular TNF levels were measured (**FIGURE 5.1**). There are no significant differences in released TNF levels by peritoneal cells from saline- and CFA-treated DP rats, or saline- and CFA-treated DR rats after culture in medium alone, or in medium containing IFN-γ. Released TNF levels by peritoneal cells from saline-treated DR rats cultured in medium containing LPS is greater than by peritoneal cells from saline-treated DP rats. Cytosolic TNF levels in peritoneal cells from saline-treated DR rats are significantly greater than those TNF levels in peritoneal cells from saline-treated DP rats cultured in medium alone ($P \leq 0.05$), in medium containing IFN-γ ($P \leq 0.05$), and in medium containing LPS ($P \leq 0.05$). There are significantly lower levels of cytosolic TNF in peritoneal cells from CFA-treated DP rats compared to peritoneal cells from CFA-treated DR rats cultured in medium alone ($P \leq 0.05$), in medium containing IFN-γ ($P \leq 0.05$), but not in medium containing LPS. The general trend that was observed in chapter 3 in which CFA treatment of DP rats increases TNF production is confirmed here.

Effect of Complete Freund's Adjuvant Treatment of Diabetes-Prone BB Rat Tumour Necrosis Factor Production by Spleen Cells After Culture In Vitro

Production of TNF by spleen cells was measured in the same animals that peritoneal cells were obtained. Spleen cells from saline- and CFA-treated DP and DR rats were cultured *in vitro* for 6 hours in medium alone, in medium containing IFN-γ, or in medium containing LPS after which time released and cytosolic TNF levels were measured (**FIGURE 5.2**). No significant differences are present in released or cytosolic TNF levels by spleen cells from saline- or CFA-treated DP and DR rats after culture in medium alone, or in medium contain IFN-γ, however. Spleen cells from saline-treated DP rats release and have cytosolic TNF levels that are significantly greater compared to spleen cells from saline-treated DR rats after culture in medium containing LPS ($P \leq 0.05$ for released and cytosolic) while both CFA-treated DP and DR rat spleen cells release similar amounts of TNF. Spleen cells from both CFA-treated DP and DR rats release significantly lower levels of TNF compared to spleen cells from saline-treated DP ($P \leq 0.05$) and DR ($P \leq 0.05$) rats. Cytosolic TNF levels in spleen cells from CFA-treated DP rats cultured in medium containing LPS are significantly lower ($P \leq 0.05$) compared to spleen cells from saline-treated DP rats.

Effect of Complete Freund's Adjuvant Treatment of Diabetes-Prone on Tumour Necrosis Factor Production by Thymocytes After Culture In Vitro

Thymocytes were obtained from the same animals as peritoneal and spleen cells. Thymocytes from saline- and CFA-treated DP and DR rats were cultured *in vitro* for 6 hours in medium alone, in medium containing Con A, or in medium containing PHA after which time released and cytosolic TNF levels were measured (**FIGURE 5.3**). Release of TNF by thymocytes from CFA-treated DP rats were significantly lower compared to

thymocytes from CFA-treated DR rats ($P \leq 0.05$), whereas there were no significant differences in released or cytosolic TNF levels by thymocytes from saline-treated DP and DR rats after culture in medium alone, in medium containing Con A, or in medium containing PHA.

DISCUSSION

Cytosolic TNF levels were measured in freshly isolated peritoneal and spleen cells, and thymocytes from the same DP and CFA-treated DP rats. No defects in peritoneal and spleen cell cytosolic TNF levels could be seen, however, cytosolic TNF levels in thymocytes from DP rats were lower than those levels in DR and CFA-treated DR rats, and CFA-treated DP rats suggesting that a defect in TNF production by DP thymocytes may be implicated in defective ontogeny.

Released and cytosolic TNF levels were measured in peritoneal cells, spleen cells, and thymocytes from the same DP and CFA-treated DP rats after stimulation *in vitro* for 6 hours. Released and cytosolic TNF levels by DP rat peritoneal cells are lower in response to stimulation with LPS, and that treatment with CFA increases TNF production to those levels observed from DR rats, a confirmation of the finding in Chapter 3.

Diabetes-prone rat spleen cell TNF levels confirmed the *in vivo* results, but contrast the *in vitro* results presented in Chapter 4; that spleen cells over-produce (released and cytosolic) TNF, and that treatment with CFA reduces those levels to that of spleen cells from DR and DR-CFA. These results suggest that TNF over-production by spleen cells may be regulated by the administration of CFA.

Released but not cytosolic TNF levels by thymocytes from DP rats after stimulation with Con A or PHA were lower, but not significantly compared to those levels by thymocytes from DR rats. Complete Freund's adjuvant did not increase DP rat thymocyte TNF levels to those levels observed with CFA-treated DR rats.

Released TNF by DP rat thymocytes appear to be lower, but the results are inconclusive. A short incubation time of 6 hours may be insufficient to demonstrate a deficiency in TNF production in response to T lymphocyte mitogens. Additional experiments involving the culture and stimulation of non-adherent versus adherent thymic-derived cells may be necessary to discern whether a defect exists at the level of the thymocyte or thymic stromal cells.

TNF production in the thymus may be important in initiating proliferation of thymocytes during ontogeny. TNF may have differential effects during positive selection in which thymocytes would proliferate in response to self-major histocompatibility complex (MHC) antigens and during negative selection in which they would be inhibited to proliferate in the context of self-MHC. Those thymocytes which do not fit this profile could likely be autoreactive in nature, and thus could be selected out to undergo TNF-mediated apoptosis through interactions with the p70-75 kDa TNF receptor (323). Therefore, one action of TNF during thymic ontogeny would be to ensure that autoreactive cells would be deleted (322). Those thymocytes which do produce TNF in response to intrathymic selective events would not undergo apoptosis and would eventually become mature $CD4^+$ thymocytes and lymphocytes (323). Tumour necrosis factor could also synergize with

interleukin 7 (IL-7) to induce expression of CD8 on thymocytes (378), a subset which is virtually absent in DP rats, and induce thymocyte IL-2Rα and IL-2Rβ expression (380), another defect associated with DP rat lymphocytes (83). These defects are associated with the inability of DP rat lymphocytes to proliferate in response to alloantigen and T lymphocyte mitogens even in the presence of exogenous IL-2 (77, 78), produce low levels of IL-2 (83), and the fail to generate $CD8^+$ cytotoxic T lymphocytes in mixed lymphocyte culture supplemented with IL-2 and IFN-γ (44).

SUMMARY

Preliminary results presented in this chapter demonstrate a generalized dysregulation of TNF characterized by a deficiency in peritoneal cells, over-production in spleen cells which may be attributed to autoreactive $CD4^+$ T lymphocytes, and a possible deficiency in thymocytes. Additional experiments will be necessary to determine whether deficient TNF production within the thymus can be attributed to the lymphocyte defects observed in DP rats and whether this defect can also be corrected by treatment with CFA.

TABLE 5.1

EFFECTS OF TREATMENT OF BB RATS WITH COMPLETE FREUND'S (CFA) ADJUVANT ON UNSTIMULATED CYTOSOLIC TNF BY DIFFERENT CELL TYPES

BB rat group	Treatment *in vivo*	TNF production pg/ 10^6 PEC	TNF production pg/ 10^6 Splenocytes	TNF production pg/ 25×10^6 Thymocytes
DP	Saline	0.060 ± 0.0	0.140 ± 0.060	0.387 ± 0.013
DP	CFA	0.065 ± 0.002	0.112 ± 0.019	1.138 ± 0.014^{*A}
DR	Saline	0.078 ± 0.009^{**B}	0.062 ± 0.001	0.625 ± 0.001
DR	CFA	0.458 ± 0.090	0.213 ± 0.086^{A}	0.565 ± 0.127

Note: Diabetes-prone (DP) and diabetes-resistant (DR) BB rats (ages 22-28 days) were injected once with saline or complete Freund's adjuvant (CFA), then 10-14 days later cells were collected and frozen at -70°C and thawed quickly to determine cellular TNF. Values are Means ± S.E. for TNF levels for 3 DP, 9 DR, 12 DP-CFA, and 12 DR-CFA rats for peritoneal cells (PEC); 9 DP, 12 DR, 12 DP-CFA and 12 DR-CFA rats for splenocytes; 3 DP, 4 DR, 4 DP-CFA and 4 DR-CFA rats for thymocytes from one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

$^{*}P < 0.05$, $^{**}P < 0.005$ for DP *vs.* DR.
$^{A}P < 0.05$, $^{B}P < 0.005$ for saline *vs.* CFA.

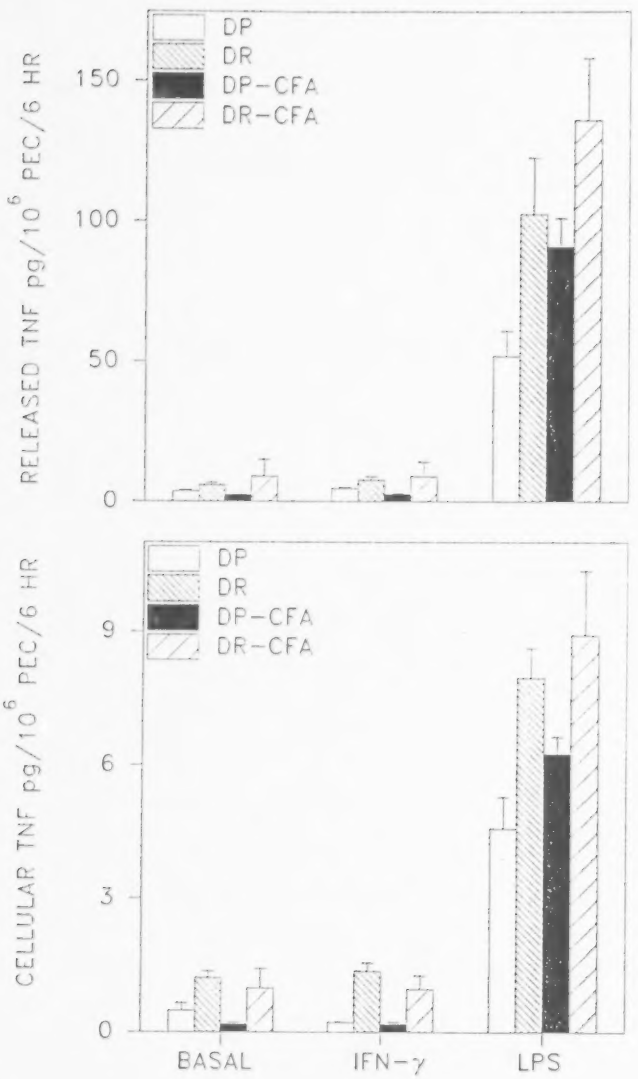

FIGURE 5.1
Tumour Necrosis Factor (TNF) Production by Peritoneal Cells From Saline- or Complete Freund's Adjuvant (CFA)-Treated Diabetes-Prone (DP) and Diabetes Resistant (DR) Rats Stimulated *In Vitro*. Peritoneal cells obtained from saline- and CFA-treated DP and DR BB rats (age 49 days) were cultured for 6 hours in medium alone, in medium containing IFN-γ (10^2 U/mL) or in medium containing LPS (0.5 ug/mL). TNF levels in cell incubation medium and cytosol (Mean ± S.E.) are shown for 4 DR, 3 DP, 4 DR-CFA, and 4 DP-CFA rats from one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

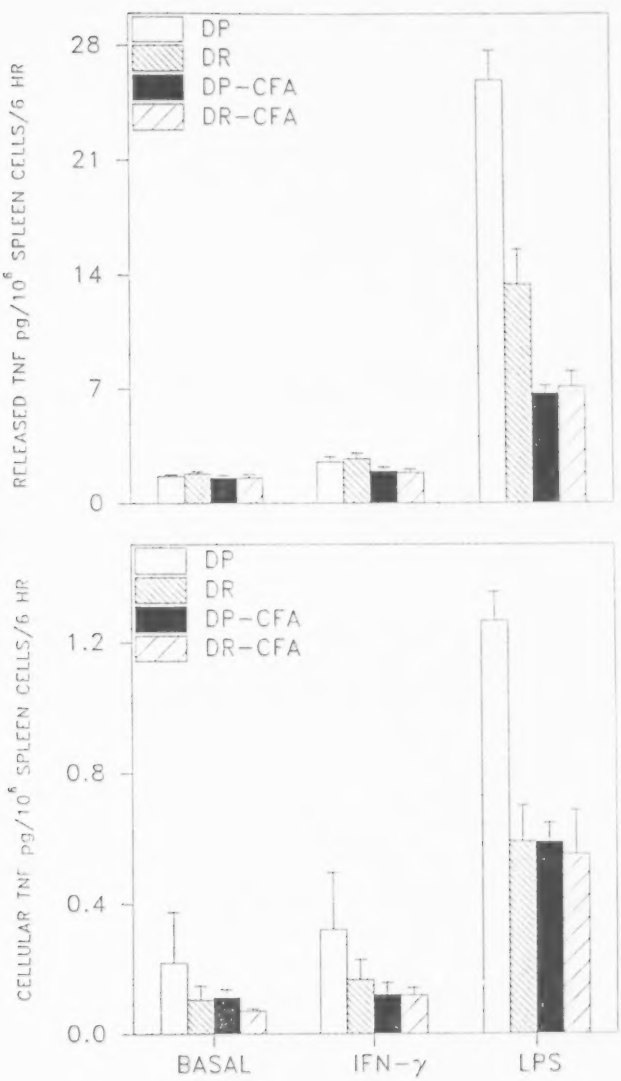

FIGURE 5.2
Tumour Necrosis Factor (TNF) Production by Spleen Cells From Saline- or Complete Freund's Adjuvant (CFA)-Treated Diabetes-Prone (DP) and Diabetes Resistant (DR) Rats Stimulated *In Vitro*. Spleen cells obtained from saline- and CFA-treated DP and DR BB rats (age 49 days) were cultured for 6 hours in medium alone, in medium containing IFN-γ (10^2 U/mL), in or medium containing LPS (0.5 ug/mL). TNF levels in cell incubation medium and cytosol (Mean ± S.E.) are shown for 4 DR, 3 DP, 4 DR-CFA, and 4 DP-CFA rats from one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

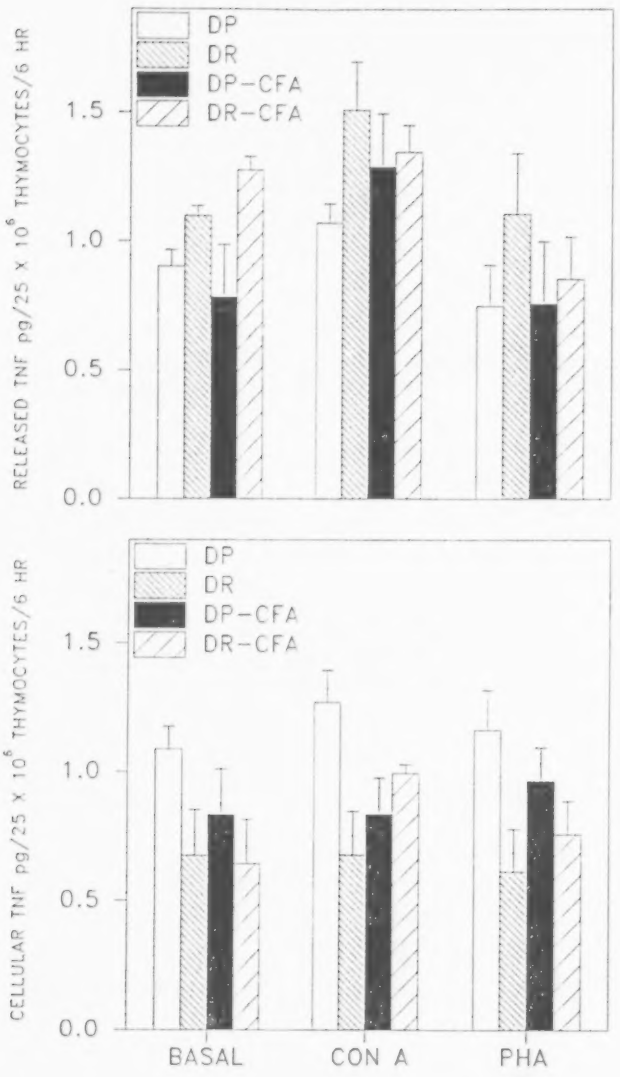

FIGURE 5.3

Tumour Necrosis Factor (TNF) Production by Thymocytes From Saline- or Complete Freund's Adjuvant (CFA)-Treated Diabetes-Prone (DP) and Diabetes Resistant (DR) Rats Stimulated *In Vitro*. Thymocytes obtained from saline- and CFA-treated DP and DR BB rats (age 49 days) were cultured for 6 hours in medium alone, in medium containing Con A (5 ug/mL), or in medium containing PHA (4.0 ug/mL). TNF levels in cell incubation medium and cytosol (Mean ± S.E.) are shown for 4 DR, 3 DP, 4 DR-CFA, and 4 DP-CFA rats from one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

CHAPTER 6

TUMOUR NECROSIS FACTOR PRODUCTION BY LYMPHOID CELLS FROM YOUNG DIABETES-PRONE RATS DIFFERS FROM OLD NON-DIABETIC DIABETES-PRONE BB RATS

INTRODUCTION

Diabetes-prone (DP) BB/Wor rats develop insulin-dependent diabetes mellitus with an incidence of 85% between 60 and 120 days of age with a frequency of less than 10% beyond 120 days of age (381, 382). As demonstrated in chapters 3, 4, and 5, TNF production was shown to be dysregulated in a tissue-specific manner. Deficient TNF production by peritoneal cells from DP rats is thought to be associated with disease development. Those DP rats that remain diabetes-free may produce levels of TNF similar to diabetes-resistant (DR) rats. To test this hypothesis, TNF production by peritoneal cells, spleen cells, and thymocytes after stimulation *in vitro* was measured in DP rats 40-49 days of age and in non-diabetic DP rats 182-220 days of age. At the same time, cytosolic, membrane-bound, and released TNF was measured to determine whether a cellular defect in TNF biosynthesis exists.

RESULTS

Tumour Necrosis Factor Production by Peritoneal Cells and Spleen Cells from Old BB Rats and Young BB Rats In Culture

In a preliminary experiment, an *in vitro* time course study was done to determine whether old non-diabetic DP rat peritoneal cells and spleen cells had levels (released and cytosolic) of TNF that were similar to DR rats.

As shown in **FIGURE 6.1**, peritoneal cells pooled from 4 young DP (DP-Y) rats released greatly decreased amounts of TNF compared to peritoneal cells pooled from 4 young DR (DR-Y) rats after culture for 3, 6, and 18 hours in medium alone, in medium containing IFN-γ, or in medium containing LPS. After culture in medium alone, released and cytosolic TNF levels by peritoneal cells pooled from 4 old DP (DP-O) rats were similar to peritoneal cells from young DR (DR-Y) and peritoneal cells pooled from 4 old DR (DR-O) rats. In medium containing IFN-γ, TNF levels from peritoneal cells from DP-O rats were similar to DR-O rats. After culture in medium containing LPS, TNF levels by peritoneal cells from DP-O rats were greater than by peritoneal cells from DR-Y rats and similar to DR-O rats.

As shown in **FIGURE 6.2**, released and cytosolic TNF levels by pooled spleen cells from 4 DP-Y rats were similar to TNF levels expressed by pooled spleen cells from 4 DP-O rats, 4 DR-Y rats and 4 DR-O rats cultured for 3, 6 and 18 hours in medium alone, in medium containing IFN-γ, or in medium containing LPS. Overall, no stark differences were seen in TNF levels (released and cytosol) by spleen cells from young and old rats.

A more extensive study was done to confirm the findings in **FIGURES 6.1 and 6.2**. Unstimulated cytosol, membrane and total (cytosol + membrane) TNF levels were measured. As shown in **TABLE 6.1**, no significant differences were seen in cytosolic TNF levels in peritoneal cells from DP-Y and DR-Y rats, and DP-O and DR-O rats, however, cytosoiic TNF levels in peritoneal cells from DP-O rats are significantly greater compared to DP-Y rats and DR-O compared to DR-Y rats. Membrane TNF levels in peritoneal cells

from DP-Y rats were greater compared to membrane TNF levels in peritoneal cells from DR-Y rats, and membrane TNF levels in peritoneal cells from DR-O rats were greater compared to DP-O rats. Membrane TNF levels in peritoneal cells from DP-Y and DP-O rats were similar while membrane TNF levels in peritoneal cells from DR-O rats were significantly greater than from DR-Y rats. Total TNF (cytosol + membrane) levels from peritoneal cells from DP-Y rats were greater than from DR-Y rats. Total TNF levels from peritoneal cells from DP-O rats were significantly less than from DR-O rat, while total TNF levels by peritoneal cells from DP-O rats were greater than from DP-Y rats, and total TNF levels by peritoneal cells from DR-O rats were greater than from DR-Y rats.

TNF levels were measured in peritoneal cells after *in vitro* culture for 6 hours in medium alone, or in medium containing LPS (**FIGURE 6.3**). Released TNF levels by unstimulated peritoneal cells from DP-Y and DP-O rats were similar and were significantly lower compared to DR-Y ($P \leq 0.05$) and DR-O ($P \leq 0.001$) rats. Released TNF levels by peritoneal cells from DR-Y rats were significantly lower compared to DR-O ($P \leq 0.05$) rats. In response to LPS stimulation, peritoneal cells from DP-Y rats released lower levels of TNF, but not significantly compared to DP-O and DR-Y rats. Released TNF levels by DR-Y rat peritoneal cells were also lower but not significantly, compared to DR-O rats.

Unstimulated cytosolic TNF levels in peritoneal cells from DP-Y rats were significantly lower compared to peritoneal cells from DP-O ($P \leq 0.05$) and DR-Y ($P \leq 0.05$) rats. Unstimulated cytosolic TNF levels in peritoneal cells from DR-Y and DR-O rats were similar while cytosolic TNF levels in peritoneal cells from DP-Y rats were significantly lower compared to DP-O ($P \leq 0.05$) rats. Unstimulated cytosolic TNF levels in peritoneal cells from DR-Y rats were significantly lower compared to DR-O ($P \leq 0.05$) rats. Cytosolic TNF levels in peritoneal cells from DP-Y rats cultured in medium containing LPS was significantly lower compared those levels in peritoneal cells from DR-Y ($P \leq 0.05$) and DP-O ($P \leq 0.05$) rats. Cytosolic TNF levels in peritoneal cells from DP-O rats were significantly lower compared to those levels in peritoneal cells from DR-O ($P \leq 0.05$) rats, and cytosolic TNF levels in peritoneal cells from DP-Y rats were lower than from DR-Y rats. Cytosolic TNF levels by DR-Y peritoneal cells were lower, but not significantly different from those levels from DR-O rats.

Finally, membrane TNF levels were measured in peritoneal cells after *in vitro* culture in medium alone or in medium containing LPS. While unstimulated membrane TNF levels in peritoneal cells from DP-Y and DP-O rats were lower, there were no significant differences compared to those levels in peritoneal cells from DR-Y and DR-O rats. Although lower, membrane TNF levels by peritoneal cells from DP-Y rats were not significantly different compared to those levels from peritoneal cells from DP-O rats. There were no significant differences in membrane TNF levels by peritoneal cells from DR-Y and DR-O rats.

Tumour Necrosis Factor Production by Spleen Cells from Old Non-Diabetic BB Rats and Young BB Rats After Culture In Vitro

Cytosolic, membrane and total (cytosolic + membrane) TNF levels were measured in freshly isolated spleen cells from young (DP-Y and DR-Y) and old (DP-O and DR-O) BB rats. As shown in **TABLE 6.2**, there are no significant differences in cytosolic TNF levels in spleen cells from DP-Y rats compared to DR-Y and DP-O rats, DP-O rats compared to DR-O rats, however, cytosolic TNF levels in spleen cells from DR-Y rats is lower than those

levels in spleen cells from DR-O rats. These findings are similar for membrane and total TNF levels, however, membrane TNF levels in spleen cells from DP-O rats are significantly greater than those levels by spleen cells from DR-O and DP-Y rats.

Spleen cell TNF levels (released, cytosol and membrane) were measured after *in vitro* culture for 6 hours in medium alone, in medium containing Con A, or in medium containing LPS (**FIGURE 6.4**). DP-O rat spleen cells cultured in medium alone released significantly greater levels of TNF compared to DR-O ($P \leq 0.05$) and DP-Y ($P \leq 0.05$) rats. There were no significant differences in cytosolic or membrane TNF levels between the four rat groups. DP-O and DR-Y rat spleen cells cultured in medium containing LPS released significantly greater levels of TNF compared to those levels by spleen cells from DR-O ($P \leq 0.05$ compared to DP-O and DR-Y rats) rats, while there were no significant differences in cytosolic or membrane TNF levels between the four rat groups. There were no significant differences in released TNF levels by spleen cells from DP-O rats and DR-O rats or DP-Y rats and DR-Y rats after culture in Con A. Released TNF levels by spleen cells from DP-O rats were significantly greater compared to those levels by spleen cells from DP-Y ($P \leq 0.05$) rats, and DR-O rat compared to DR-Y ($P \leq 0.05$) rats. No significant differences in cytosolic and membrane TNF levels by spleen cells were seen between DP-O and DR-O rats, or DP-Y and DR-Y rats, however, cytosolic TNF levels in spleen cells from DR-O rats were significantly greater than those levels in spleen cells from DR-Y ($P \leq 0.05$) rats.

Tumour Necrosis Factor Production by Thymocytes from Old Non-Diabetic BB Rats and from Young BB Rats After Culture In Vitro

Cytosolic, membrane and total (cytosolic + membrane) TNF levels were measured in freshly isolated thymocytes from young (DP-Y and DR-Y) and old (DP-O and DR-O) rats (**TABLE 6.3**). Cytosolic TNF levels in thymocytes from DP-Y rats were significantly greater than those levels in thymocytes from DR-Y ($P \leq 0.05$) rats, while no significant differences in cytosolic TNF levels in thymocytes were seen in DP-O and DR-O rats or DR-O and DR-Y rats. No significant differences were seen in membrane and total TNF in thymocytes from the four rat groups.

Thymocyte TNF levels (released, cytosol and membrane) were measured after *in vitro* culture for 6 hours in medium alone, in medium containing Con A, or in medium containing LPS (**FIGURE 6.5**). No significant differences in released, cytosolic, or membrane TNF levels were seen in the four rat groups except released TNF by thymocytes from DR-O rats was greater compared to those levels by thymocytes from DP-O ($P \leq 0.05$) and DR-Y ($P \leq 0.05$) rats after culture in medium alone. No significant differences in thymocyte TNF levels (released and cytosol) were seen after culture in medium containing LPS. Membrane TNF levels in thymocytes from DP-Y rats were significantly lower compared to levels in thymocytes from DP-O ($P \leq 0.05$) rats, and levels by thymocytes from DR-Y rats compared to levels in thymocytes from DR-O ($P \leq 0.05$) rats. No significant differences in released, cytosolic, or membrane TNF levels were seen in thymocytes cultured in medium containing Con A except for released TNF levels by thymocytes from DR-O rats compared to levels by thymocytes from DR-Y ($P \leq 0.05$) rats.

Tumour Necrosis Factor Levels in Unstimulated Peritoneal Cells and Sera from BB Rats and other Rat Strains

In a separate series of experiments, unstimulated TNF levels were measured in peritoneal cells or in sera of different rats strains. A shown in **TABLE 6.4**, no significant differences could be seen in TNF levels (cytosolic, membrane and total) in freshly isolated DP rat peritoneal cells when compared to other rat peritoneal cells except that cytosolic and total (cytosolic + membrane) TNF levels by peritoneal cells from DP-Y rats were significantly less when compared to levels by peritoneal cells from DP-O rats, and cytosolic, membrane and total TNF levels by peritoneal cells from DR-Y rats were significantly lower compared to those levels by peritoneal cells from DR-O rats. In addition, cytosolic and membrane TNF levels in peritoneal cells from the WF rat, which shares the same MHC haplotype as BB rats, were similar to levels by peritoneal cells from DP and DR rats, while total TNF levels by peritoneal cells from WF rats were significantly lower compared total TNF levels by peritoneal cells from DP-O and DR-O rats.

After culture in medium alone, released, membrane and total (released + cytosolic + membrane) TNF levels by peritoneal cells from DP rats were significantly lower compared to DP-O rats, while cytosolic and total (released + cytosolic + membrane) TNF levels by peritoneal cells were significantly lower in DR-Y rats compared to DR-O rats (**TABLE 6.5**). There were no significant differences in unstimulated TNF levels (released, cytosolic, membrane, total) by peritoneal cells from DP and DR rats compared to CFA-treated DP and DR rats, or to Wistar Furth and Lewis rats.

Serum TNF levels from unstimulated DP and DR rats were measured and compared to other rat strains (**TABLE 6.6**). Serum TNF values ranged from 1.65 pg/mL to 3.47 pg/mL and were not significantly different between the groups.

DISCUSSION

In a preliminary experiment, TNF production (released and cytosolic) by old non-diabetic DP rat peritoneal cells after stimulation *in vitro* was similar to young or old DR rat peritoneal cells, and greater than by young DP rat peritoneal cells. No differences were seen with spleen cells. A more extensive study was done in which cytosolic and membrane TNF levels were measured in freshly isolated cells. Cytosolic and total (cytosolic + membrane) TNF levels by young DP rat peritoneal cells were lower compared to old DP rats. Unstimulated release of TNF by old DP rat peritoneal cells were similar to young DP rats, however, LPS-stimulated release was similar to young DR rats suggesting that those DP rats which escaped from developing diabetes may produce sufficient amounts of TNF. Cytosolic TNF levels from young DP rats was significantly lower compared to old DP rats while membrane levels from both young and old DP rats were similar and lower compared to young and old DR rats. Overall, these results suggest that peritoneal cells from old DP rats have the ability to produce greater levels of TNF that may be important in preventing diabetes. Furthermore, these results suggest that threshold levels of TNF may be necessary in preventing diabetes. This conclusion is supported by the findings that treatment of DP rats with TNF (329), OK-432 (262), CFA (271), low doses of IL-1 (285, 286), or with IL-2 (288), all of which activate macrophages and stimulate TNF production, prevents diabetes development, and that administration of TNF which also prevents WF islet xenograft rejection in C57BL/6J mice (328). These agents stimulate sufficient threshold levels of TNF which that can act as an immunomodulator to suppress

cellular immune function. As a result, islet grafts are not rejected and diabetes is prevented in DP rats.

TNF was measured in freshly isolated spleen cells from unstimulated young and old non-diabetic DP rats and DR rats. Membrane and total (cytosolic + membrane) TNF levels were greater in old DP rat spleen cells compared to young DP and DR rat spleen cells. Spleen cell TNF production was measured after stimulation *in vitro*. No significant difference were seen between the four groups in response to Con A or LPS. As discussed in chapter 5, a short culture time of 6 hours may be insufficient to determine whether a defect or over-production exists. Because TNF was measured by bioassay, it was not possible to determine which TNF species is (are) present and which is (are) most abundant. To a limited extent, this may be determined by separating the spleen cell subsets and determining their relative levels of TNF. However, the L929-8 TNF bioassay does not discriminate between TNF-α and LTα, and does not detect LTβ. Since TNF production by peritoneal cells is predominantly TNF-α and is increased in old non-diabetic DP rat peritoneal cells, whole spleen cell TNF levels in these cells may contain a larger amount of TNF-α compared to young DP rat spleen cells. Splenic macrophage TNF-α production may be immuno-regulatory in a manner similar to administration of TNF (320), or through the induction of other cytokines such as IL-10 (366).

No real differences in cytosolic, membrane or total (cytosolic + membrane) TNF in freshly isolated thymocytes between the four groups could be seen. After 6 hours of stimulation *in vitro* with Con A or LPS, no real differences could be seen. These results again suggest that 6 hours of culture may be insufficient to determine whether TNF production is deficient or over-produced by thymocytes from DP rats.

The opportunity was taken to compare TNF levels in freshly isolated peritoneal cells from untreated DP and DR rats, from CFA-treated DP and DR rats, from old DP and DR rats and from WF and Lewis rats. No real differences could be seen between the eight groups except that in all cases both old DP and DR peritoneal cell TNF levels were greater, even compared to CFA-treated rats but not after culture for 6 hours in medium alone. Instead, both old DP and DR rat peritoneal cell TNF levels were greater than their respective young counterparts. The opportunity was also taken to examine unstimulated serum TNF levels in these groups. While the levels varied, no real differences could be observed.

The results presented here suggest that macrophage TNF production is one factor which may predict whether DP rats become susceptible to developing diabetes. Additional experiments could be done to determine the role of TNF in disease susceptibility. Such experiments would involve obtaining peritoneal lavages from a random sampling of at least 24 DP rats at 20-30 days of age, 120 days of age and 200 days of age, to measure TNF production. Those animals that yield the greatest peritoneal cell TNF levels would be considered as candidates that are least likely to develop diabetes. Other experiments would involve purifying spleen and thymocyte subsets to determine whether a deficiency or over-production exists.

These results also indicate that while overall TNF production (released, cytosolic and membrane) is deficient in DP rat peritoneal cells, but not spleen cells or thymocytes, there is not likely a defect in the processing of the TNF molecule. Rather, TNF production is

repressed by young DP rat peritoneal cells. To determine whether this repression occurs at the level of transcription or post-transcriptionally, molecular studies would be needed to determine relative TNF transcript abundance. Normal transcript numbers would suggest that deficient TNF production occurs through a post-transcriptional block, while deficient numbers of transcripts would suggest that repression occurs either at the level of signal transduction, at the level of transcription, or in mRNA stability. Lastly, addition of neutralizing antibodies to suppressor cytokines such as IL-4, IL-10 and members of the prostaglandin family during culture would help to identify if any or all of these molecules participate suppressing peritoneal cell TNF production.

Lastly, because cytosolic and membrane TNF levels in freshly isolated young DP rat peritoneal cells and after culture *in vitro* in medium alone, appear similar to DR and CFA-treated animals, this suggests that upon stimulation of TNF production, there may also be stimulation of suppressor molecules. Again, the addition of neutralizing antibodies to specific cytokines will help determine the cause of deficient TNF production by DP rat peritoneal cells.

SUMMARY

Tumour necrosis factor production by peritoneal cells from DP rats that did not become diabetic were greater than of young DP rats and similar to DR rats, suggesting that a threshold level of TNF may be important in preventing diabetes. The results are inconclusive with respect to spleen cells and thymocyte TNF production and require additional experiments with increased culture times to determine which subsets produce TNF and whether a deficiency or over-production exists. Finally, freshly isolated peritoneal cells and peritoneal cells cultured in medium alone from young DP rats produce similar levels of TNF compared to young DR rats and other rats strains suggesting that TNF production is repressed rather than defective. Additional experiments are necessary to determine whether this repression occurs at the receptor level, at the level of signal transduction, transcriptionally, or post-transcriptionally.

TABLE 6.1

TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES

BB rat group	TNF (pg/10^6 cells)		
	Cytosol	Membrane	Total
DP-YOUNG	0.066 ± 0.025[A]	0.236 ± 0.033[*]	0.313 ± 0.021[*A]
DR-YOUNG	0.043 ± 0.002[A]	0.085 ± 0.023[B]	0.187 ± 0.034[A]
DP-OLD	0.218 ± 0.046	0.350 ± 0.094[*]	0.657 ± 0.122[*]
DR-OLD	0.372 ± 0.066	0.726 ± 0.079	1.244 ± 0.133

Note: Values are Means ± S.E. for TNF levels in cell cytosol and membrane fractions of peritoneal macrophages from 4 young (age 49 days) DP and DR BB rats and 4 old DP and 3 old DR (age 220 days) BB rats in one experiment.

[*] $F < 0.05$ compares DR to DP rats.
[A] $P < 0.05$, [B] $P < 0.0001$ compares YOUNG to OLD rats.

TABLE 6.2

TNF LEVELS IN UNSTIMULATED SPLEEN CELLS FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES

BB rat group	TNF (pg/10^6 cells)		
	Cytosol	Membrane	Total
DP-YOUNG	0.049 ± 0.005	0.042 ± 0.002	0.134 ± 0.007
DR-YOUNG	0.041 ± 0.001	0.041 ± 0.023	0.127 ± 0.068
DP-OLD	0.057 ± 0.006	0.059 ± 0.003^{*A}	0.161 ± 0.007^{*A}
DR-OLD	0.050 ± 0.002^{A}	0.047 ± 0.002	0.137 ± 0.002

Note: Values are Means ± S.E. for TNF levels in cell cytosol and membrane fractions of whole spleen cells from 4 young (age 49 days) DP and DR BB rats and 4 old DP and DR (age 220 days) BB rats in one experiment.

$^{*}P < 0.05$ compares DR to DP rats.
$^{A}P < 0.05$ compares YOUNG to OLD rats.

TABLE 6.3

TNF LEVELS IN UNSTIMULATED THYMOCYTES FROM DIABETES-PRONE (DP) AND DIABETES-RESISTANT (DR) BB RATS OF DIFFERENT AGES

BB rat group	TNF (pg/25 X 10^6 cells)		
	Cytosol	Membrane	Total
DP-YOUNG	0.068 ± 0.007*A	0.064 ± 0.006	0.179 ± 0.013
DR-YOUNG	0.040 ± 0.001	0.063 ± 0.007	0.147 ± 0.009
DP-OLD	0.050 ± 0.005	0.061 ± 0.009	0.159 ± 0.012
DR-OLD	0.040 ± 0.0	0.087 ± 0.0	0.167 ± 0.0

Note: Values are Means ± S.E. for TNF levels in cell cytosol and membrane fractions of whole thymocytes from 4 young (age 49 days) DP and DR BB rats and 4 old DP and 1 old DR (age 220 days) BB rats in one experiment.

*$P < 0.05$ compares DR to DP rats.
A$P < 0.05$ compares YOUNG to OLD rats.

TABLE 6.4

TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIFFERENT RAT STRAINS

	TNF (pg/0.5 X 10^6 cells)		
rat group	Cytosol	Membrane	Total
DP	0.33 ± 0.09	1.41 ± 0.31	1.74 ± 0.34
DR	0.44 ± 0.16	1.17 ± 0.38	1.62 ± 0.41
DP-CFA	0.56 ± 0.07	1.35 ± 0.38	1.91 ± 0.41
DR-CFA	0.61 ± 0.16	1.36 ± 0.38	1.97 ± 0.40
DP-OLD	2.95 ± 0.33[B]	2.39 ± 0.28	5.34 ± 0.57[B]
DR-OLD	3.56 ± 0.78[B]	2.56 ± 0.44[A]	6.11 ± 1.12[B]
WF	0.27 ± 0.04	0.63 ± 0.16	0.93 ± 0.16[*]
LEWIS	0.67 ± 0.30	0.99 ± 0.31	1.67 ± 0.35

Note: Values are Means ± S.E. for TNF levels in cell cytosol, membrane, and total (cytosol + membrane) fraction of peritoneal cells for 6 rats of each strain from one experiment.

[*]$P < 0.001$ for BB rats compared to other strains.
[A]$P < 0.05$, [B]$P < 0.005$ for DR and DP rats compared to DR-O and DP-O rats.

TABLE 6.5

TNF LEVELS IN UNSTIMULATED PERITONEAL CELLS FROM DIFFERENT RAT STRAINS AFTER CULTURE *IN VITRO*

rat group	TNF (pg/0.5 X 10^6 cells/6 hr)			
	Released	Cytosol	Membrane	Total
DP	2.19 ± 0.35	1.23 ± 0.40	1.00 ± 0.19	4.42 ± 0.52
DR	2.06 ± 0.48	0.62 ± 0.12	2.51 ± 0.83	4.65 ± 1.04
DP-CFA	2.55 ± 0.49	0.37 ± 0.12	0.71 ± 0.22	3.63 ± 0.28
DR-CFA	4.49 ± 2.12	0.69 ± 0.32	1.28 ± 0.38	6.46 ± 2.29
DP-OLD	3.86 ± 0.49*	1.33 ± 0.31	3.91 ± 2.22**	6.85 ± 0.65*
DR-OLD	4.05 ± 0.80	2.21 ± 0.31**	2.28 ± 0.49	8.55 ± 1.35*
WF	5.85 ± 2.18	1.13 ± 0.54	0.76 ± 0.37	7.74 ± 3.06
LEWIS	3.36 ± 0.87	0.52 ± 0.20	0.54 ± 0.17	4.42 ± 1.21

Note: Values are Means ± S.E. for TNF levels in the medium, cell cytosol, membrane, and total (medium + cytosol + membrane) fractions of peritoneal cells for 6 DR, 6 DP-CFA, 6 LEWIS, 6 Wistar Furth (WF), 6 DR-OLD, and 6 DP-OLD rats, 5 DP and 5 DR-CFA rats from one experiment.

*$P < 0.05$, ** $P < 0.01$ for DR and DP rats compared to DR-OLD and DP-OLD rats.

TABLE 6.6

SERUM TNF LEVELS FROM DIFFERENT RAT STRAINS (UNSTIMULATED)

Rat group	TNF (pg/mL serum)
DP	2.31 ± 0.41
DR	2.40 ± 0.52
DP-CFA	1.65 ± 0.12
DR-CFA	3.47 ± 0.84
DR-OLD	ND
DP-OLD	ND
WF	2.51 ± 0.70
LEWIS	1.66 ± 0.17

Note: Values are Means ± S.E. for serum TNF levels from 6 rats/strain in one experiment.

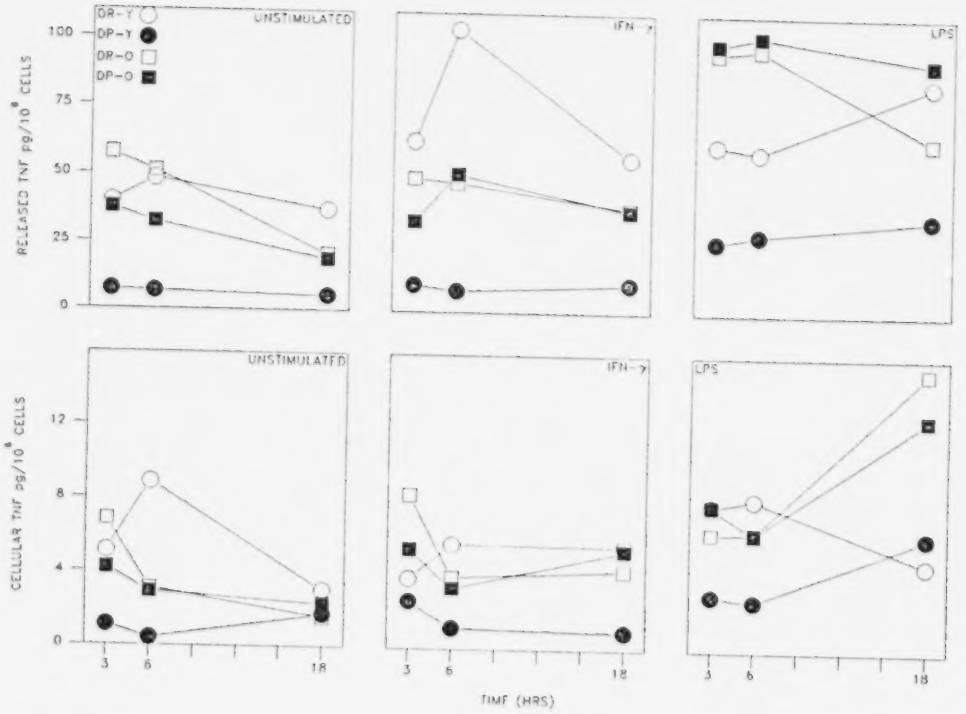

FIGURE 6.1

TNF Production by Peritoneal Cells From Young and Old DR and DP Rats after Stimulation *In Vitro*. Peritoneal cells obtained from young DP and DR (DP-Y and DR-Y; ages 49 days) and old non-diabetic DP and DR (DP-O and DR-O; ages 170 days) were cultured for 3, 6 and 18 hours in complete medium alone, in medium containing IFN-γ (10^2 U/mL), or in medium containing LPS (0.5 ug/mL) as described in *Materials and Methods*. TNF levels in cell incubation medium and cell cytosol are shown for pooled cells from three animals/group in one experiment.

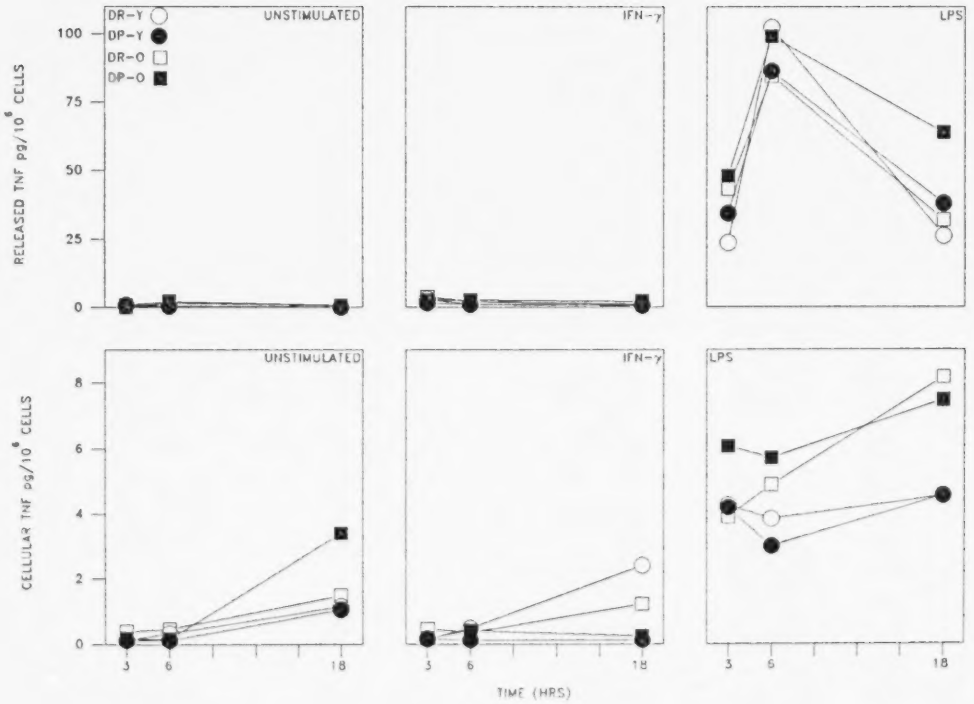

FIGURE 6.2

TNF Production by Spleen Cells From Young and Old DR and DP Rats after Stimulation *In Vitro*. Spleen cells obtained from young DP and DR (DP-Y and DR-Y; ages 49 days) and old non-diabetic DP and DR (DP-O and DR-O; ages 170 days) were cultured for 3, 6 and 18 hours in complete medium alone, in medium containing IFN-γ (10^2 U/mL), or in medium containing LPS (0.5 ug/mL) as described in *Materials and Methods*. TNF levels in cell incubation medium and cell cytosol are shown for pooled cells from three animals/group in one experiment.

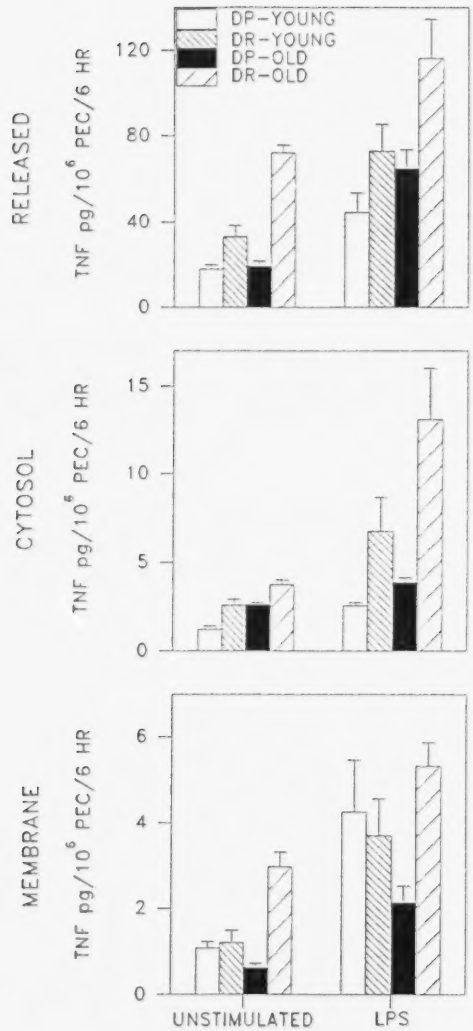

FIGURE 6.3

Peak TNF Production by Peritoneal Cells From Young and Old DR and DP Rats after Stimulation *In Vitro*. Peritoneal cells obtained from young DP and DR (DP-Y and DR-Y; ages 40 days) and old non-diabetic DP and DR (DP-O and DR-O; ages 170 days) were cultured for 6 hours in complete medium alone (basal), in medium containing IFN-γ (10^2 U/mL), or in medium containing LPS (0.5 ug/mL) as described in *Materials and Methods*. TNF levels in cell incubation medium, cell cytosol, and cell membrane (Mean ± S.E.) are shown for 4 DP-Y, 4 DR-Y, 3 DP-O and 3 DR-O rats in one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

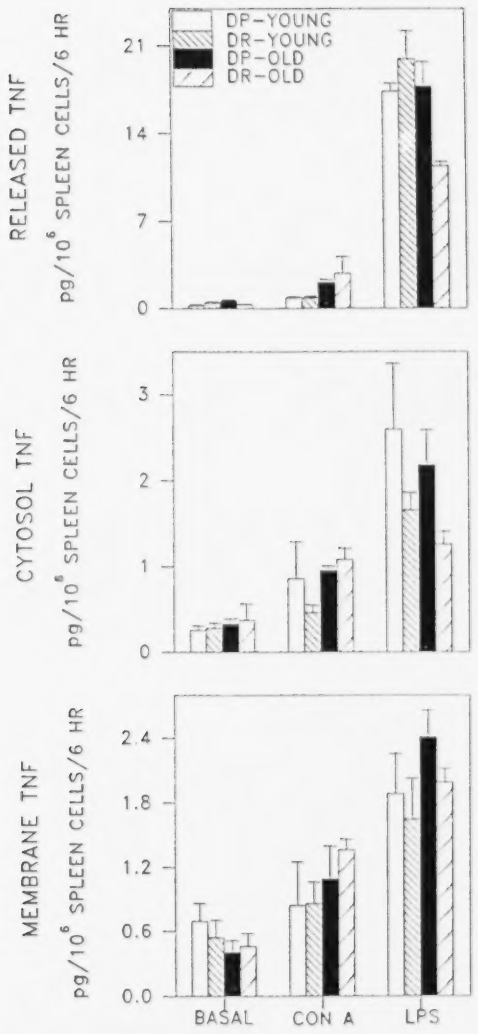

FIGURE 6.4

TNF Production by Spleen Cells From Young and Old DR and DP Rats after Stimulation *In Vitro*. Spleen cells obtained from young DP and DR (DP-Y and DR-Y; ages 40 days) and old non-diabetic DP and DR (DP-O and DR-O; ages 170 days) were cultured for 6 hours in complete medium alone (basal), in medium containing Con A (5 ug/mL), or in medium containing LPS (0.5 ug/mL) as described in *Materials and Methods*. TNF levels in cell incubation media, cell cytosol, and cell membrane (Mean ± S.E.) are shown for 4 DP-Y, 4 DR-Y, 4 DR-O, and 4 DP-O rats in one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

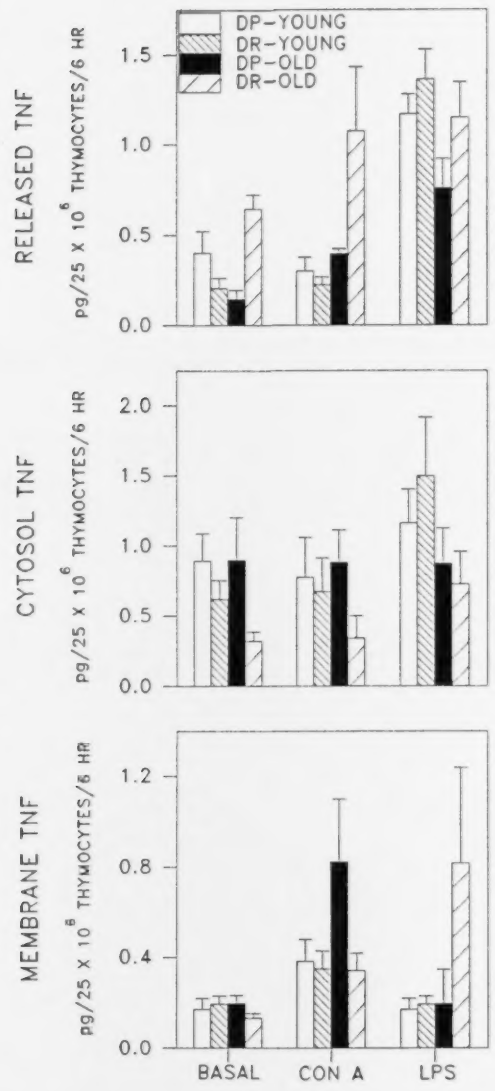

FIGURE 6.5

TNF Production by Thymocytes From Young and Old DR and DP Rats after Stimulation *In Vitro*. Thymocytes obtained from young DP and DR (DP-Y and DR-Y; ages 40 days) and old non-diabetic DP and DR (DP-O and DR-O; ages 170 days) were cultured for 6 hours in complete medium alone (basal), in medium containing Con A (5 ug/mL), or in medium containing LPS (0.5 ug/mL) as described in *Materials and Methods*. TNF levels in cell incubation media, cell cytosol, and cell membrane (Mean ± S.E.) are shown for 4 DP-Y, 4 DR-Y, 4 DR-O, and 4 DP-O rats in one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

CHAPTER 7

SERUM TUMOUR NECROSIS FACTOR RESPONSES ARE INCREASED IN DIABETES PRONE BB RATS: A POSSIBLE RELATION TO DEFICIENT CORTICOSTERONE RESPONSES

INTRODUCTION

Insulin-dependent diabetes in the BB rat is a polygenic disease involving lymphoid cells and their soluble polypeptide products, cytokines. Cytokines have been demonstrated to be cytotoxic islet cells *in vitro* and include interleukin 1 (IL-1), tumour necrosis factor (TNF), lymphotoxin (LT), and interleukin 6 (IL-6)(142-159). However, diabetes-prone (DP) BB rats chronically treated with IL-1, TNF, or LT are protected against disease development (270, 285, 286, 300, 328, 329, 334, 335), as are those animals which are treated with adjuvants such as OK-432, a streptococcal cell wall product (262), or complete Freund's adjuvant (CFA)(236).

Tumour necrosis factor, IL-1 and IL-6 production which can be induced by macrophages after stimulation with endotoxin or lipopolysaccharide (LPS) can in turn stimulate endocrine function associated with the hypothalamic-pituitary-adrenal (HPA) axis (383). In particular, these cytokines stimulate increased production of HPA-associated endocrine products culminating with adrenocorticotropic hormone (ACTH) and corticosterone (383). Glucocorticoids can feedback negatively on the macrophage to suppress cytokine synthesis (384). Together, cytokines and endocrine products constitute the immune-HPA (IHPA).

More recently, defects within the IHPA have been implicated with autoimmune disease. LEWIS rats develop a form of rheumatoid arthritis after injection with streptococcal cell wall (SCW), and a form of multiple sclerosis, experimental allergic encephalomyelitis (EAE) after injection with a myelin basic protein (MBP)-CFA admixture (385, 386). Both of these autoimmune diseases are thought to occur, in part, as a result of a defect in corticotropin-releasing hormone production (385, 386). Obese strain (OS) chickens develop a form of spontaneous Hashimoto's thyroiditis which is associated with increased amounts of corticosteroid-binding globulin (CBG) and a deficiency in plasma corticosterone levels (387).

Animal models of autoimmune diseases attributed to defects within the immune-hypothalamic-pituitary axis (IHPA), may be the result of faulty negative-feedback networks. These same faulty networks may extend to the BB rat model of human insulin-dependent diabetes mellitus. To test the hypothesis that a defect in the IHPA exists and contributes to diabetes development in the BB rat, serum TNF and corticosterone levels were measured in DP and diabetes-resistant (DR) rats at various times after stimulation *in vivo*. This study was extended to determine whether CFA-protected DP rats had increased corticosterone levels.

RESULTS

Tumour Necrosis Factor in the Serum of Diabetes-Prone (DP) and Diabetes-Resistant BB Rats After Stimulation In Vivo

In preliminary experiments, serum TNF levels were measured in DP and DR rats after stimulation *in vivo*. DP and DR rats were stimulated *in vivo* by intravenous injection of saline, IFN-γ (10^5 U), LPS (500 ug) or IFN-γ followed by LPS as described in *Materials and Methods*. The scatter plot shows the results of these preliminary experiments (**FIGURE 7.1**). No significant differences in serum TNF levels were seen after stimulation *in vivo* with saline, IFN-γ, or LPS. Serum TNF levels do appear to be somewhat greater for DP rats compared to DR rats after stimulation with IFN-γ + LPS and serum TNF values for DP rats appear to be skewed into two populations.

Serum TNF and Corticosterone Levels in Different Rat Strain After Stimulation with Lipopolysaccharide (LPS)

Serum TNF and corticosterone levels in DP, DR, LEWIS and WF rats were measured after stimulation *in vivo* with PBS (**FIGURE 7.2**) or with LPS (**FIGURE 7.3**). Serum corticosterone levels were measured in samples provides to Dr. S. Harvey, Department of Physiology, University of Alberta. In response to PBS, serum TNF levels were not significantly different in the rat groups, however, DR corticosterone levels were significantly greater 0.5 and 1 hour after activation compared to DP ($P \leq 0.01$ for both 0.5 and 1 hour). Serum TNF levels were not significantly different between the four rat groups 0.5 hours and 1 hour after LPS stimulation, whereas serum TNF levels were significantly greater for WF rats compared to DR ($P \leq 0.01$) and DP ($P \leq 0.01$) rats, LEWIS rats compared to DP rats ($P \leq 0.01$), and DR rats compared to DP rats ($P \leq 0.05$) after 2 hours after stimulation with LPS. Serum corticosterone levels were significantly greater for DR rats compared to DP ($P \leq 0.01$) rats 0.5 hours after LPS stimulation while no significant differences were seen between the other groups. Serum corticosterone levels were significantly greater for LEWIS rats compared to DP rats 1 hour ($P \leq 0.05$) and 2 hours ($P \leq 0.05$) after LPS stimulation. No other significant differences were seen at 1 hour. Two hours after LPS stimulation, LEWIS corticosterone levels were significantly greater compared to DR rats ($P \leq 0.05$), and serum corticosterone levels from WF rats were greater than DP rats ($P \leq 0.01$).

In a second experiment, serum TNF and corticosterone levels were measured in DP, DR, LEWIS, and WF rats after stimulation *in vivo* with LPS (**FIGURE 7.4**). However, in this experiment, the rats were sacrificed and exsanguinated differently from those presented in Figures 7.2 and 7.3. No significant differences in 0 hour serum TNF levels were observed between DR, DP, WF and LEWIS rats. DR rat serum corticosterone levels were significantly greater compared to serum corticosterone levels from DP rats ($P \leq 0.05$), WF rats ($P \leq 0.05$), and LEWIS rats ($P \leq 0.05$). At 0.5 hours after LPS stimulation *in vivo*, serum TNF levels from DR rats were significantly greater than levels from LEWIS ($P \leq 0.01$) and WF ($P \leq 0.01$) rats, while levels from DP rats were significantly greater than from WF rats ($P \leq 0.05$). There were no significant differences in serum corticosterone levels between the 4 rat groups at 0.5 hours. At 1 hour post-LPS stimulation, DR rat serum TNF levels were significantly greater than LEWIS ($P \leq 0.0001$) and WF ($P \leq 0.0001$) rats, and DP rat serum TNF levels compared to LEWIS ($P \leq 0.001$), but not WF rats. No significant differences were seen in serum corticosterone levels between the 4 rat groups at this time point. Serum TNF levels from DP rats were significantly greater compared to LEWIS ($P \leq 0.0005$), and DR ($P \leq 0.01$) rats 2 hours after stimulation *in vivo* with LPS, and while

greater than WF rat serum TNF levels, was not significant. Serum TNF levels from DR rats were significantly greater than from LEWIS rats ($P \leq 0.05$), while serum TNF levels from WF rats were significantly greater than both DR ($P \leq 0.0005$) and LEWIS ($P \leq 0.0001$) rats. No significant differences in serum corticosterone levels could be seen between the 4 rat groups at this time point.

Serum TNF and corticosterone levels were measured in DP, DP-CFA, DR, and DR-CFA rats stimulated *in vivo* with LPS (**FIGURE 7.5**). Serum TNF levels from DR-CFA rats were significantly greater than serum TNF levels from DP-CFA rats at 0 hours ($P \leq 0.05$). At 0.5 hours after LPS stimulation, serum TNF levels from DP rats were significantly greater than serum TNF levels from DP-CFA rats ($P \leq 0.05$). No significant differences were seen in the other rat groups at 0.5 hours or for the four groups at 1 and 2 hours after LPS stimulation. Serum corticosterone levels from DR-CFA rats were significantly greater than serum corticosterone levels from DP-CFA rats at 0 hours ($P \leq 0.05$) and 0.5 hours ($P \leq 0.05$) after LPS stimulation. No significant differences between the other rat groups were seen at 0 and 0.5 hours after LPS stimulation, and no significant differences were seen at 1 and 2 hours after LPS stimulation for the 4 rat groups.

To determine whether peritoneal cells TNF levels contribute to serum TNF levels, peritoneal cells obtained from 0 and 1 hour time point rats were cultured *in vitro* for 3 hours. Released, cytosolic and membrane TNF levels were measured (**FIGURE 7.6**). Released TNF levels by unstimulated peritoneal cells were similar for the 4 rat groups although TNF levels by peritoneal cells from DR rats were greater. A significant difference in membrane TNF levels from peritoneal cells were observed between DR and DP rats ($P \leq 0.05$), and in cytosolic TNF levels in peritoneal cells from DR rats compared to LEWIS rats ($P \leq 0.05$). In response to *in vivo* stimulation with LPS, released TNF levels by peritoneal cells from DR and DP rats were significantly greater than LEWIS ($P \leq 0.01$ for DR and $P \leq 0.05$ for DP) and greater than WF ($P \leq 0.05$ for DR and DP). Cytosolic TNF levels in peritoneal cells from DR rats were significantly greater than in peritoneal cells from LEWIS rats ($P \leq 0.05$) and membrane TNF levels compared to WF rats ($P \leq 0.01$). No other significant differences were observed.

DISCUSSION

In a preliminary experiment, serum TNF levels were compared between DP and DR BB rats after stimulation *in vivo* with interferon-γ (IFN-γ), lipopolysaccharide (LPS) or the combination. No significant differences could be seen between DR and DP rats after injection of PBS. However, increases in serum TNF levels could be seen after injection of IFN-γ, LPS, or the combination.

In a second experiment, serum TNF and corticosterone levels were measured in DP, DR, Wistar Furth (WF), and LEWIS rats at different times after injection of PBS or LPS. In response to PBS, serum TNF levels were similar in all four animal groups, however, serum corticosterone levels in DR rats were greater at 0.5 and 1.0 hours than those levels in DP rats. Serum corticosterone levels differed among the four rat groups in response to PBS injection. However, it is not likely the PBS itself, but rather, the anaesthetizing procedure itself stressed the rats, resulting in the increased corticosterone levels. These animals were anaesthetized by subcutaneous injection of Somnotol and the handling and injection of Somnotol itself may be responsible for the physical stress resulting in increased serum

corticosterone levels, at least in DR and WF rats (388). In response to LPS, serum TNF levels were not different between the four groups early (0.5 and 1.0 hours), while both LEWIS and WF rat serum TNF levels were greater than DR and DP rat serum TNF levels at the 2.0 hour time point. Serum corticosterone levels from DP rats did not change throughout the treatment time whereas DR rat serum corticosterone levels were greater early (0.5 and 1.0 hours) and decreased in contrast to both LEWIS and WF. From this experiment, it appears that TNF released into the serum can have a positive effect on the induction of corticosterone, but not in DP rats.

Another experiment was done with efforts to minimize any external stressor that may affect serum cytokine and corticosterone levels. Animals were not anaesthetized after the LPS injection time points, but instead, were decapitated thereby eliminating the possible artificial increases in serum corticosterone associated with handling and injection of Somnotol. No significant differences were seen in serum TNF levels at 0 hours between the four rat groups, whereas serum corticosterone levels were greater from DR rats compared to the other rat groups. Serum TNF levels were greater in DR rats than LEWIS and WF, but not DP at the 0.5 hour time point and both DR and DP were greater than LEWIS and WF at the 1.0 hour time point. In contrast, no differences were seen in corticosterone levels at 0.5 hours. Only DR corticosterone levels were greater than LEWIS and WF levels at 1.0 hours. At 2.0 hours post-LPS treatment, serum TNF levels were greatest in DP rats followed by WF rats and finally DR rats. However, the pattern of serum TNF levels by DP rats appears not to plateau compared to WF rats. Serum corticosterone levels by DR rats are greater from 0 and 0.5 hours, and peaked at 1.0 hour compared to the other rats. In DP, LEWIS and WF rats, this peak occurred at 0.5 hours. A reduction in serum TNF levels is only evident from WF and LEWIS rats. Overall, these results suggest that corticosterone production in DR rats can regulate release of TNF into the serum. However, corticosterone production in DP rats appears low compared to both the DR rat and to the MHC-matched WF rat and is similar to the LEWIS rat. This may help to explain the apparent dysregulation of TNF production and the excessive release of TNF into the serum of DP rats. Furthermore, it appears that like the OS chicken and the LEWIS rat, that defects within the immune-hypothalamic-pituitary axis may contribute to the susceptibility of diabetes in DP rats (384-386).

The effect of CFA on serum TNF and corticosterone levels in DR and DP rats was investigated. Unstimulated serum TNF levels from CFA-treated DR rats were greater than those levels from CFA-treated DP rats, however serum corticosterone levels were greater in CFA-treated DP rats than CFA-treated DR rats. At later time points, serum TNF levels after LPS administration were similar except at 0.5 hours in which serum TNF levels were greater in DP rats. No differences in serum corticosterone levels were present at later time points. Therefore, it is not likely that CFA regulates serum corticosterone production.

It has been reported that physical stress promotes diabetes in BB rat with an increase in incidence and at an earlier age (389). This is in accord with a second report in which diabetes in BB rats was decreased by low dose cortisone treatment (308). Together, these studies suggest that physical stress which is an inducer of corticosterone production may be detrimental to DP rats by reducing further, an already impaired TNF response, similar to low dose cortisone treatment. From the results presented above and cited literature, corticosterone production appears to be, to a limited extent, impaired in DP rats. This

could result in the observed excessive serum TNF levels at later time points after LPS administration.

It has been reported that a steroid transport protein, termed corticosteroid binding globulin (CBG) is defective in DP rats. This defect is attributed to a point mutation whereby a methionine at position 276 is substituted by isoleucine. This substitution results in a reduced binding affinity for corticosterone to 66% of that Wistar rats and an increased rate of dissociation of corticosterone from CBG (304). Together, an apparent impaired production of corticosterone combined with a defective steroid transport protein may also contribute to DP rat diabetes development. It is interesting to note that DR rat serum corticosterone levels are in excess when compared to DP and WF rats. While no reports are available describing a mutation in DR rat CBG, this defect may none-the-less be present as well. In order to compensate, DR rats may have developed a mechanism by which over-production of corticosterone increases the ability of the defective CBG to transport corticosterone to the target cell, and down-regulate macrophage cytokine production including TNF.

Finally, released, cellular and membrane TNF levels were measured in unstimulated peritoneal cells from DR, DP, WF and LEWIS rats, and after stimulation *in vivo* with LPS for 1 hour. Released TNF by both DR and DP rat peritoneal cells are greater compared to WF and LEWIS rats, and DR is greater compared to DP rats. These results suggest that one cell population that may contribute to the serum TNF levels observed are peritoneal cells. However, other cell sources of TNF should not be discounted and include blood neutrophils (390), spleen cells including T and B lymphocytes and splenic macrophages (391) and endothelial cells (392). The endothelial cells is a good candidate since it has been recently shown that these cells express CD14, one of a family of LPS receptors. Since endothelial cells line the surface of blood vessels, LPS can directly induce cytokine release into the blood and serum.

Diabetes-resistant BB rats appear to release excessive amounts of corticosterone into the serum. It would be of interest to determine whether DR rats, like DP rats also have a mutant form of CBG, and to determine whether excessive corticosterone production is compensatory. To address this question, two experiments could be done. The first experiment would be to adrenalectomize the DR rats and follow them for diabetes development. The second experiment would be to adrenalectomize DR rats and deplete the lymphoid cells of $RT6^+$ cells and follow them for diabetes development.

SUMMARY

The results presented here suggest that corticosterone production by DP rats may be defective (deficient) and when combined with a defective corticosterone binding globulin may be unable to regulate (suppress) release of TNF into the serum by suppressing macrophages and other cell sources of TNF. In addition, DR rats have elevated serum levels of corticosterone which may act to suppress release of TNF into the serum. There also appears to be no direct effect of CFA on either corticosterone production or release of TNF into the serum. Finally, peritoneal cells may be one cell subset that contributes to the release of TNF into the serum. These results suggest that like other animal models of autoimmune disease, diabetes susceptibility in BB rats may also be associated with defects within the immune-hypothalamic-pituitary axis.

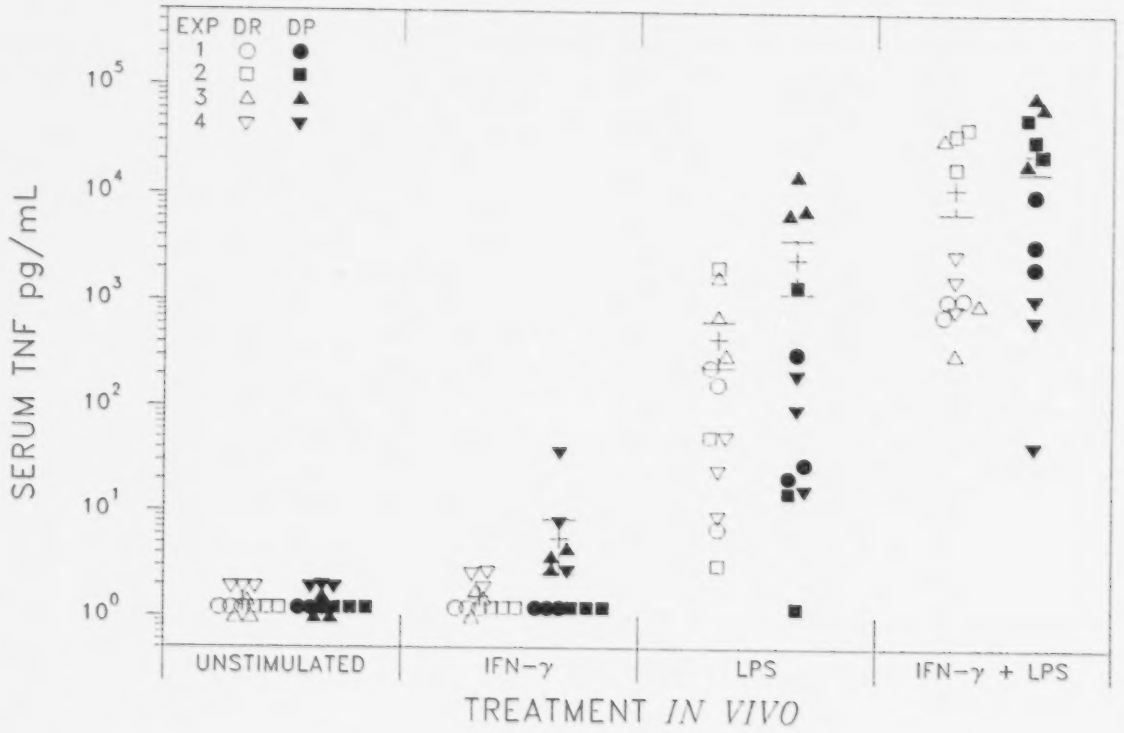

FIGURE 7.1
Serum TNF Levels in Diabetes-Resistant (DR) and Diabetes-Prone (DP) Rats Stimulated *In Vivo*. DR and DP BB rats (ages 23-45 days) were injected intravenously with saline, interferon-γ (IFN-γ, 10^5 U), lipopolysaccharide (LPS, 500 ug) or IFN-γ + LPS as described in *Materials and Methods*. Four hours later, blood was drawn and sera prepared. Serum TNF levels (Means ± S.E.) are shown for 12 DR and 12 DP rats from 4 matched experiments.

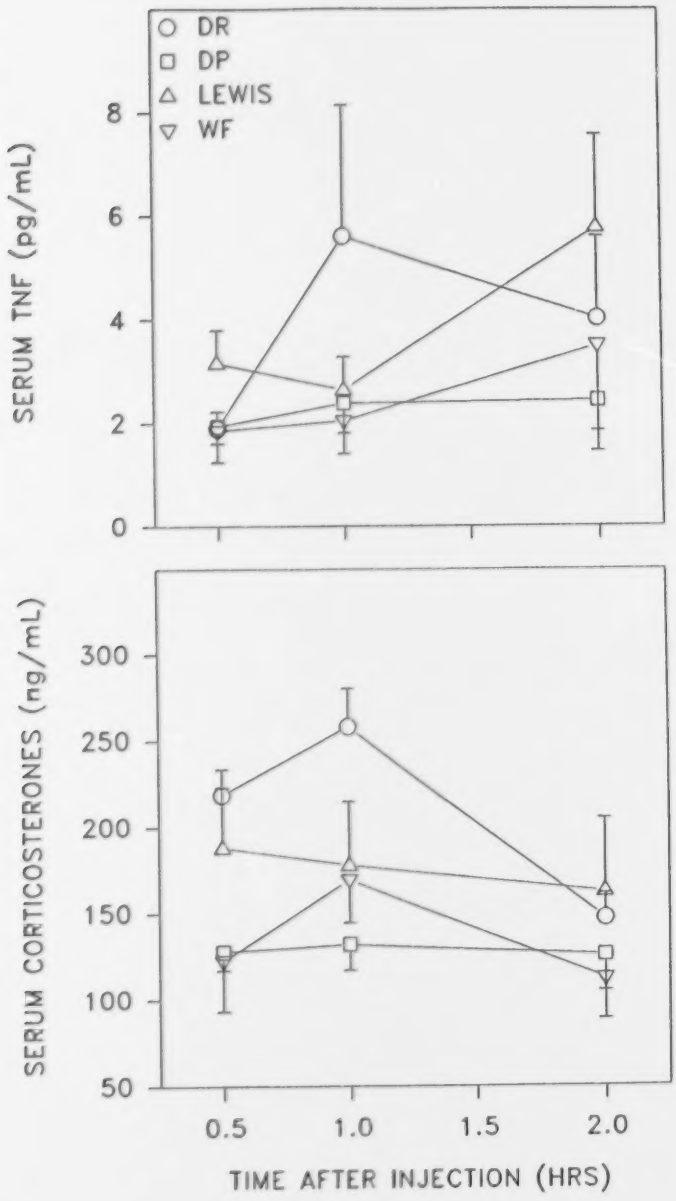

FIGURE 7.2
Serum TNF and Corticosterone Levels in Diabetes-Resistant (DR), Diabetes-Prone (DP), LEWIS and Wistar Furth (WF) Rats Stimulated *In Vivo*. DR, DP, LEWIS, and WF rats (ages 29-43 days) were injected intravenously with saline as described in *Materials and Methods*. At 0.5, 1.0, 1.5 and 2.0 hours, blood was drawn and sera prepared. Serum TNF and corticosterone levels (Mean ± S.E.) are shown for 6 DR, 6 DP, 6 LEWIS, and 6 WF rats for each time point in 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

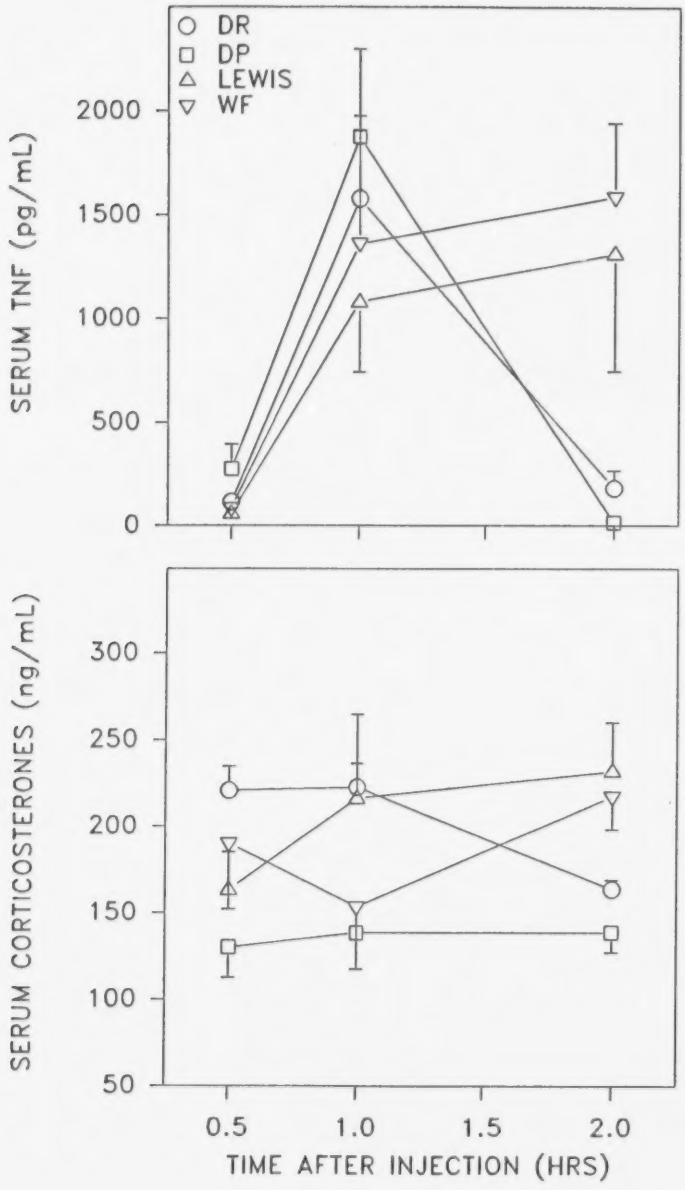

FIGURE 7.3
Serum TNF and Corticosterone Levels in Diabetes-Resistant (DR), Diabetes-Prone (DP), LEWIS and Wistar Furth (WF) Rats Stimulated *In Vivo*. DR, DP, LEWIS, and WF rats (ages 29-43 days) were injected intravenously with lipopolysaccharide (LPS 500 ug) as described in *Materials and Methods*. At 0.5, 1.0, 1.5 and 2.0 hours, blood was drawn and sera prepared. Serum TNF and corticosterone levels (Mean ± S.E.) are shown for 6 DR, 6 DP, 6 LEWIS, and 6 WF rats for each time point in 2 matched experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

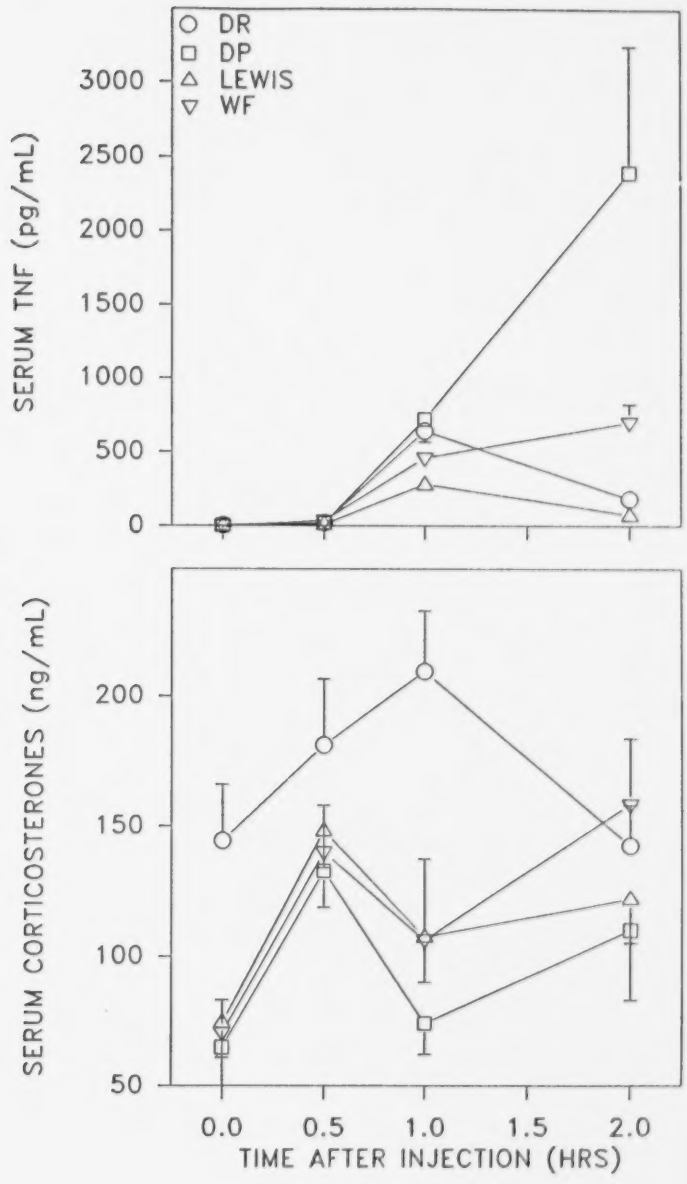

FIGURE 7.4
Serum TNF and Corticosterone Levels in Diabetes-Resistant (DR), Diabetes-Prone (DP), LEWIS and Wistar Furth (WF) Rats Stimulated *In Vivo*. DR, DP, LEWIS, and WF rats (ages 40-45 days) were injected intravenously with lipopolysaccharide (LPS 500 ug) as described in *Materials and Methods*. At 0, 0.5, 1.0, 1.5 and 2.0 hours, blood was drawn and sera prepared. Serum TNF and corticosterone levels (Mean ± S.E.) are shown for 9 to 12 DR, DP, LEWIS, and WF rats for each time point in two experiments. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

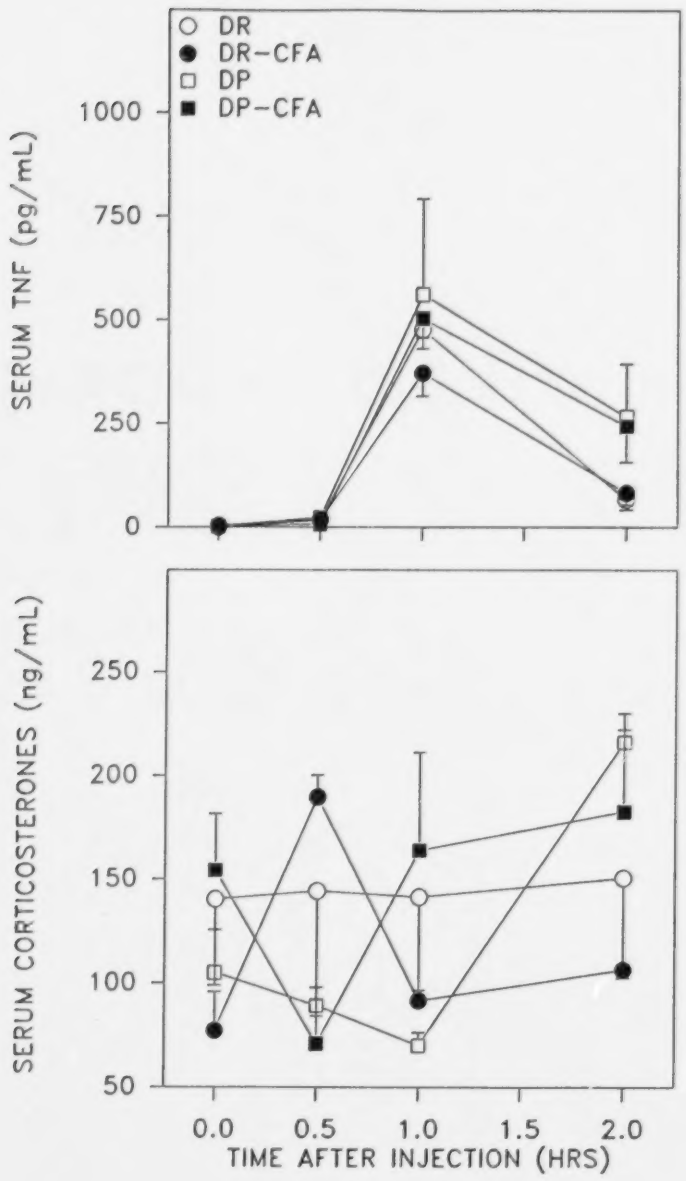

FIGURE 7.5
Serum TNF and Corticosterone Levels in Diabetes-Resistant (DR), Diabetes-Prone (DP), and Complete Freund's Adjuvant (CFA)-Treated DP and DR Rats Stimulated *In Vivo*. DR, DP, DR-CFA, and DP-CFA rats (ages 40-45 days) were injected intravenously with lipopolysaccharide (LPS 500 ug) as described in *Materials and Methods*. At 0, 0.5, 1.0, 1.5 and 2.0 hours, blood was drawn and sera prepared. Serum TNF and corticosterone levels (Mean ± S.E.) are shown for 3 to 6 DR, DP, DP-CFA, and DR-CFA rats for each time point in one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

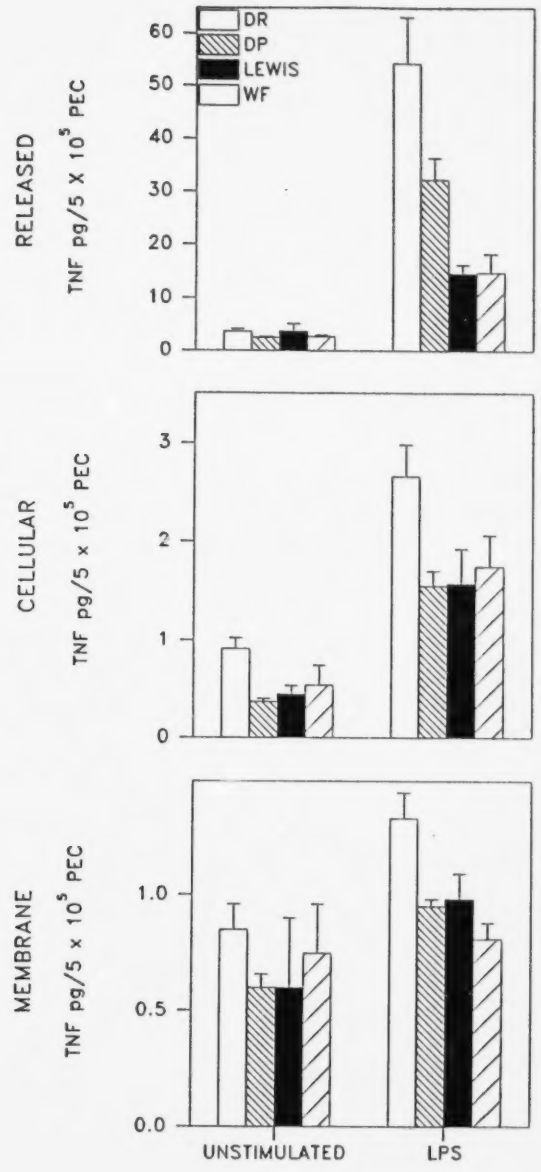

FIGURE 7.6

TNF Production by Peritoneal Cells From Diabetes-Resistant (DR), Diabetes-Prone (DP), LEWIS and Wistar Furth (WF) Rats Stimulated *In Vivo*. Peritoneal cells were obtained from 0 hour and 1 hour injection times described in Figure 7.4, and were cultured for 3 hours in complete medium as described in *Materials and Methods*. TNF contents in culture media (released), cell cytosol, and cell membrane (Mean ± S.E.) are shown for 6 rats per group per condition from one experiment. Statistical significance was determined by the Mann-Whitney Test for unpaired samples.

CHAPTER 8

POLYCLONAL ANTISERUM TO TUMOUR NECROSIS FACTOR REVERSES COMPLETE FREUND'S ADJUVANT-MEDIATED PROTECTION AGAINST DIABETES DEVELOPMENT IN DIABETES-PRONE BB RATS

INTRODUCTION

The BB rat is an animal model of human insulin-dependent diabetes mellitus (1, 2). Diabetes development can be prevented by immunosuppressive and immunoregulatory therapies. Included in the genre of immunosuppressive therapies are bone marrow transplantation (193-197, 200), neonatal thymectomy and or thymic transplantation (201, 204, 205), lymphocyte transfusion (207, 208, 211, 212), chronic administration of antibodies to cell surface proteins and cell lymphocyte subsets (214-216, 219-221), and pharmacological agents (226-233). Immunoregulatory therapies which prevent diabetes include mitogens and lectins (263, 264), adjuvants (232, 236, 261, 265-272, 275-277, 280), and cytokines (270, 280, 285, 286, 289, 300, 311, 328-330, 334, 335).

In chapter 3, it was demonstrated that tumour necrosis factor (TNF) production by diabetes-prone (DP) BB rat peritoneal cells was deficient, and treatment with complete Freund's adjuvant (CFA), while protecting against diabetes development, also increased DP rat peritoneal cell TNF production. On the other hand, spleen cell TNF levels in DP rats are excessive, and treatment of DP rats with CFA reduces these excessive TNF levels. However, it is not known whether CFA is protective by increasing peritoneal cell TNF levels directly or whether it acts through other mechanisms. To address the hypothesis that CFA protects against diabetes development by increasing peritoneal cell TNF production, CFA-protected DP rats were treated with a polyclonal antisera to TNF and followed for diabetes development.

RESULTS

Effects of a Rabbit Polyclonal Antisera on TNF-Mediated Cytotoxicity of L929-8 Cells.

Rabbit polyclonal antiserum to TNF was prepared. Prior to, and during the immunization procedures, sample bleeds were obtained and neutralizing activity against murine TNF-α-mediated cytotoxicity of L929-8 cells was measured. As shown in **TABLE 8.1**, sera from five different rabbits demonstrated no neutralizing activity prior to immunization. Four days after the last immunization, rabbit sera were again tested for their neutralizing activity against TNF-mediated cytotoxicity of L929-8 cells. Serum from rabbits 1, 3 and 4 were capable of inhibiting TNF-mediated L929-8 cell killing by 100% with dilutions ranging from 0.06-529.3 X 10^3, and 50% killing with dilutions ranging from 0.3-314.6 X 10^3.

Presence of Semi-Purified Rabbit Antisera to TNF in Rat Sera

TABLE 8.2 shows the specific neutralizing activity of semi-purified polyclonal antibodies to TNF in comparison to a commercial source. Pooled sera from rabbits' 1 and 4 were 10-fold more potent than the commercial source. After 5 injections of semi-purified TNF antisera, sera from experimental DP rats demonstrated the presence of specific neutralizing activity against TNF as determined by a specific ELISA and ranged from 1 to 2.7 X 10^4 specific neutralizing units.

Neutralization of TNF-Mediated Killing of L929-8 Cells by Serum from Anti-TNF Antiserum-Injected Rats

Anti-TNF activity in sera from experimental rats were tested in the L929-8 TNF bioassay (**TABLE 8.3**). Neutralization of nearly 100% of 50 pg/mL of TNF in the L929-8 bioassay was achieved using sera from experimental rats 1 and 2 at 1: 100 dilution, and greater than 60% neutralization by sera from rats 3 and 4.

Effect of Antiserum to TNF-α on CFA-Mediated Protection Against Diabetes in Diabetes-Prone BB Rats

In a preliminary experiment, CFA did not protect against diabetes (64% *vs.* 63% for PBS-treated control DP rats). Treatment with semi-purified antisera to murine TNF-α to CFA-treated DP rats decreased diabetes incidence to 43%, however, this was not statistically significant as determined by the Fisher Exact Test (**FIGURE 8.1**).

The experiment was repeated by Dr. Rabinovitch and his laboratory staff. The results in **FIGURES 8.2, 8.3 and 8.4** were generously lent to me for continuity of my thesis and are not results generated as part of my own thesis research. Complete Freund's adjuvant protected against diabetes in DP rats by reducing the incidence of diabetes to 50%. This protection was reversed with chronic administration of a sheep antisera to murine TNF and resulted in an increase in incidence of diabetes to 75%, similar to PBS-treated controls (80%). The combination of CFA and control sheep sera resulted in an incidence of diabetes of 44%, not significantly different from CFA-treated rats (50%).

Effect of Tumour Necrosis Factor on Diabetes in Diabetes-Prone BB Rats

Daily administration of 20 ug TNF-α to DP rats protects against diabetes development resulting in 7 of 19 (37%) becoming diabetic compared to PBS-treated rats in which 15 of 18 (83%) became diabetic by 110 days of age. These results were statistically significant ($P \leq 0.01$) as determined by the Fisher Exact Test.

Effect of Sheep Antiserum to TNF Against Diabetes in Diabetes-Prone BB Rats

Diabetes-prone BB rats were treated chronically with a sheep antisera to murine TNF to determine whether antisera alone could affect diabetes development in DP rats. The results in **FIGURE 8.4** show that sheep antisera to TNF does not influence diabetes development in DP rats and resulted in an incidence of diabetes of 82%, similar to PBS-treated (92%), or PBS + control sera-treated rats (71%). No statistical differences were observed between the experimental and control animal groups as determined by the Fisher Exact Test.

DISCUSSION

Complete Freund's adjuvant (CFA) protects against diabetes development in DP BB rats. However, the mechanism(s) by which CFA protects against disease is not completely understood. While it was shown that CFA-treated DP rat peritoneal cells had increased TNF levels compared to untreated DP rats, it is unknown whether this increase in TNF production contributes directly or indirectly to prevention of diabetes.

In a preliminary experiment, CFA-treated DP rats were treated with an antisera to murine TNF or with normal rabbit sera starting at 26 days of age. The results are inconclusive in this experiment since CFA alone did not mediate any form of protection against diabetes

development. One explanation for this lack of CFA-mediated protection may have been the route of treatment. These animals were treated through the footpads as opposed to the traditional peritoneal route. Footpad protection was problematic and not reliable since it has been observed previously that treatment by this route did not increase peritoneal cell TNF levels (results not shown). One explanation for the protective effect of intraperitoneal administration of CFA is the proximity of both the spleen and pancreas. Macrophages from the peritoneum may traffic to, and extravasate into the spleen and pancreas to mediate a suppressor effect. In contrast, macrophages from peripheral lymph nodes such as popliteal lymph nodes may not traffic to either of these organs, and therefore not mediate any suppressor effect. Administration of TNF antisera would either have no effect or may worsen the already deficient status of the DP rat. Furthermore, there was anti-TNF activity in the antisera since whole antisera and semi-purified sera did display significant TNF-neutralizing activity. The semi-purified antisera used for treatment had a 10-fold greater specific neutralizing activity against TNF-α than did reference antisera. In addition, serum samples from experimental animals prepared 3 days after administration of the last dose displayed between 63 and 93% neutralizing activity against 50 pg/mL recombinant murine TNF-α.

In a repeat experiment, CFA was administered intraperitoneally rather than in the footpad prior to treatment with sheep TNF antisera. In this experiment, TNF antisera was administered starting at 80 days of age, and reversed the protection observed with CFA resulting in an incidence of diabetes similar to PBS-treated rats. These results suggest that one mechanism by which may CFA protect against diabetes development is to act through increased systemic TNF production. The administration of CFA may act in a manner similar to that observed in a companion experiment in which the administration of TNF-α protein directly reduces the incidence of diabetes. This further supports the notion that CFA prevents diabetes by increasing peritoneal cell TNF production.

These results are in accord with other studies. Non-obese diabetic (NOD) mice treated with wheat flour lipopolysaccharide (wLPS) had the same level of protection against diabetes when compared to TNF-α administration (263). In addition, NOD mice treated with a streptococcal cell wall preparation, OK-432, while protecting against diabetes, also increased serum TNF-α levels (269, 270). Administration of serum from NOD mice prevented diabetes in naive NOD mice comparable to recombinant TNF and this protection could be blocked by the simultaneous treatment with an anti-TNF antibody (269). In addition, myeloid-like cells from NOD mice treated with CFA, had modest increases in TNF-α production (275). In contrast to the protective effects of mitogens, lectins, and adjuvants which increase TNF production, it was demonstrated that neonatal administration of anti-TNF-α antibodies prevents rather than induces diabetes (332). Furthermore, neonatal treatment with TNF-α protein induces rather than protects against diabetes (332). It is possible that neonatal administration of anti-TNF-α antibodies may suppress thymic ontogeny to a level in which autoreactive lymphocytes undergo apoptosis during either positive or negative selection and as a result never enter the periphery.

Additional experiments may be necessary to understand the role of TNF in the prevention of diabetes. These experiments could include: the administration of adjuvants, TNF-α or anti-TNF-α antibodies followed by phenotyping the changes in thymocyte, lymphocyte and monocyte/macrophage subsets compared to controls. This would allow for one to

determine whether TNF-α plays a positive role in lymphoid cell ontogeny and in maturation/differentiation of monocytes/macrophages to the suppressor phenotype. To define the effects of TNF in preventing diabetes, mutant forms of TNF-α which bind specifically to TNF-R can be employed to determine whether increased amounts of TNF preferentially bind to, and mediate activation through one or the other of these receptors. Lastly, cytokines known to be induced by TNF can be examined in TNF- or CFA-treated animals, or anti-TNF-α antibody-treated animals in order to determine whether a defect in TNF production results in a generalized defect in other cytokines.

SUMMARY

The results presented here confirm the observation presented in chapter 3 in which DP rat peritoneal cells produce reduced amounts of TNF, and that administration of CFA while protecting against diabetes, increases peritoneal cell TNF levels from DP rats. Furthermore, the results presented here are in accord with the results presented in chapter 5, whereby non-diabetic DP rats produce sufficient threshold amounts of TNF. Finally, administration of CFA is as effective in preventing diabetes in DP rats as the administration of recombinant TNF, and that CFA-mediated protection can be reversed by the administration of an antisera to TNF. These results suggest that TNF is important in preventing diabetes.

TABLE 8.1

NEUTRALIZATION OF MURINE TNF-α ACTIVITY ON L929-8 CELLS BY RABBIT SERUM

RABBIT	TITRE (DILUTION^{-1} X 10^3) PRE-IMMUNE[A]	POST-IMMUNE[B] 100%	50%
1	0.0	8.19	196.6
2	0.0	0.06	0.3
3	0.0	529.3	314.6
4	0.0	131.1	262.1
5[C]	0.0	0.0	0.0

[A] Pre-immunization bleed.
[B] Post-immunization period; 100% and 50% neutralization.
[C] Control immunized rabbit.

TABLE 8.2

DETECTION OF ANTIBODIES TO MURINE TNF-α IN RABBIT ANTISERUM AND IN SERA OF DP RATS TREATED WITH RABBIT ANTISERUM TO MURINE TNF-α

SOURCE	RABBIT Ig (mg/mL)	ANTI-TNF-α Ig (U/mL)	SPECIFIC NEUTRALIZING ACTIVITY AGAINST TNF-α (pg/mL)(X 10^4)
RABBIT[A]			
1 + 4	33.78	10.000	400.00
5	14.04	0.001	0.05
TNF-antisera	18.85	1.000	40.00
RAT[B]			
1	0.29	0.050	2.10
2	0.29	0.070	2.70
3	0.16	0.030	1.00
4	0.26	0.040	1.60
5	0.01	0.000	-
6	0.72	0.000	-
7	0.13	0.000	-
8	0.09	0.000	-

[A]After $(NH_4)_2SO_4$ precipitation, antiserum from rabbits' 1 and 4 were pooled and a commercial source of rabbit anti-murine TNF-α antiserum.
[B]Sera was obtained 3 days (rats 1, 2, 5, 6) and 5 days (rats 3, 4, 7, 8) from randomly selected experimental (1-4) and control-treated (5-8) rats after 5 injections of antiserum.

TABLE 8.3

NEUTRALIZATION OF MURINE TNF-α ACTIVITY AGAINST L929-8 CELLS BY SERA OF DP RATS TREATED WITH RABBIT ANTISERUM TO MURINE TNF-α

SOURCE[A]	DILUTION	O.D[B]	% NEUTRALIZATION 50 pg/mL TNF-α[C]
1 + 4	1:100	0.419	104
	1:200	0.358	89
TNF-	1:1000	0.429	123
antiserum	1:2000	0.409	117
RAT			
1	1:100	0.328	93
2	1:100	0.361	96
3	1:100	0.281	74
4	1:100	0.257	63

[A] Anti-TNF activity was assessed from $(NH_4)_2SO_4$ precipitated rabbit serum (1 + 4), from a commercial source of TNF-α antiserum and from DP rats injected with 1 + 4.

[B] Optical density of viable L929-8 cells after culture with 50 pg/mL of murine TNF-α and dilutions of antiserum.

[C] Physiological dose of TNF-α.

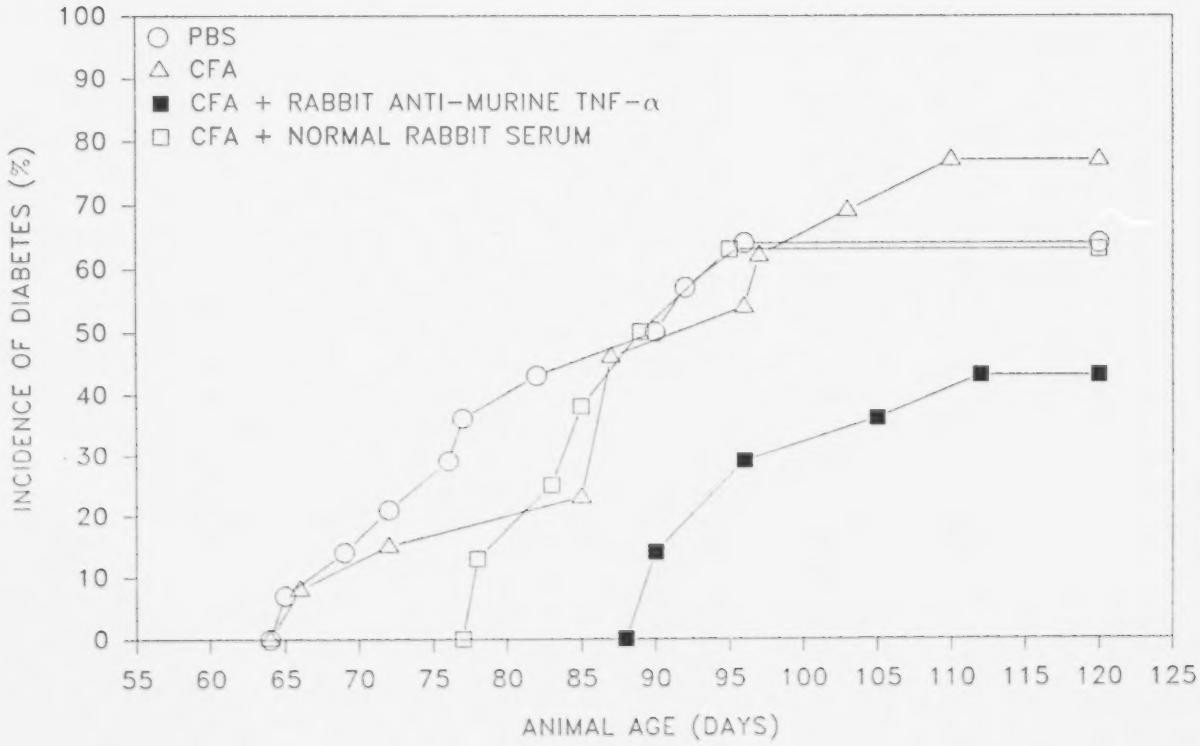

FIGURE 8.1
Effect of Rabbit Antiserum to Murine TNF-α on Complete Freund's Adjuvant-Protected Diabetes-Prone BB Rats. CFA-treated DP rats were treated chronically with PBS, rabbit anti-murine TNF-α antiserum, or normal rabbit serum 3 times a week from days of age to 120 days of age as described in *Materials and Methods*. Results are shown for 14 rats injected in the hind footpad with PBS, 13 rats injected in the hind footpad with CFA, 14 CFA + rabbit anti-murine TNF-α-antiserum-treated, and 8 CFA + normal rabbit serum-treated rats in one experiment. Statistical significance was determined by the Fisher Exact Test.

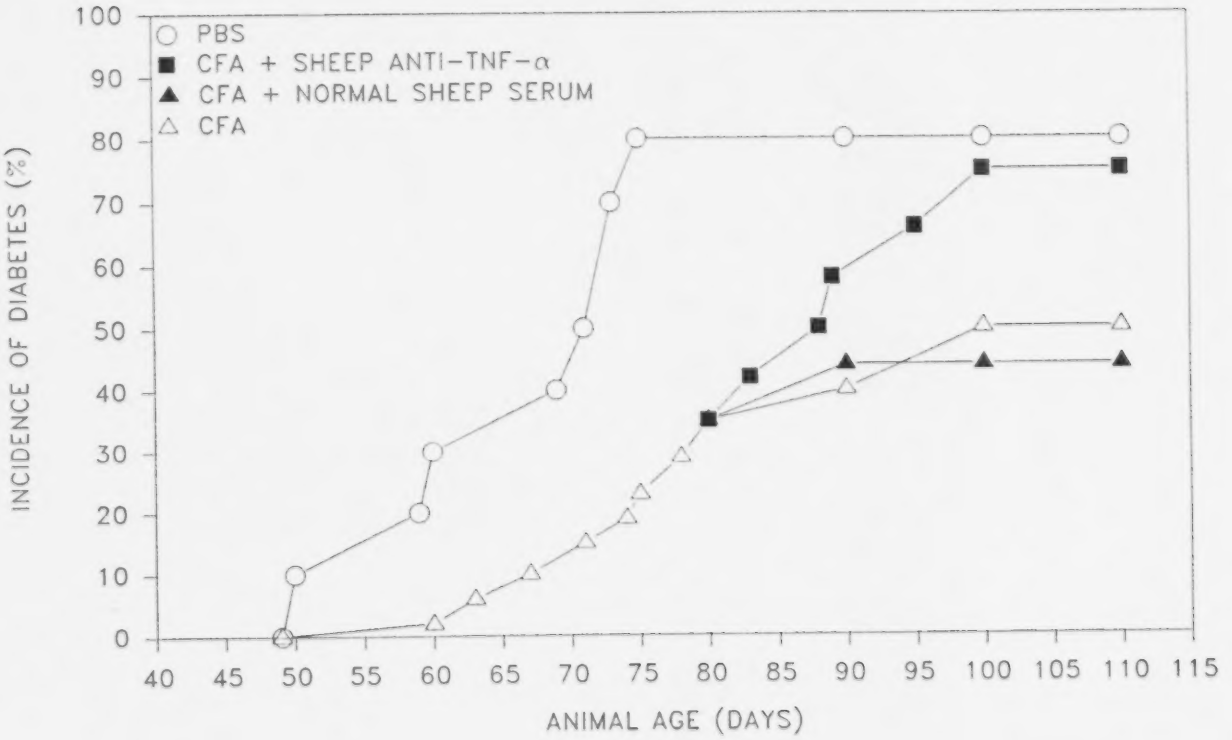

FIGURE 8.2
Effect of Sheep Antiserum to Murine TNF on Complete Freund's Adjuvant-Protected Diabetes-Prone BB Rats. CFA-treated DP rats were treated chronically with PBS, sheep anti-murine TNF antiserum, or control serum 3 times a week from 81 days of age to 110 days of age as described in *Materials and Methods*. Results are shown for 10 rats injected intraperitoneally with PBS, 10 rats injected intraperitoneally with CFA, 12 CFA + sheep anti-murine TNF-treated, and 9 CFA + control serum-treated rats in one experiment. Statistical significance was determined by the Fisher Exact Test.

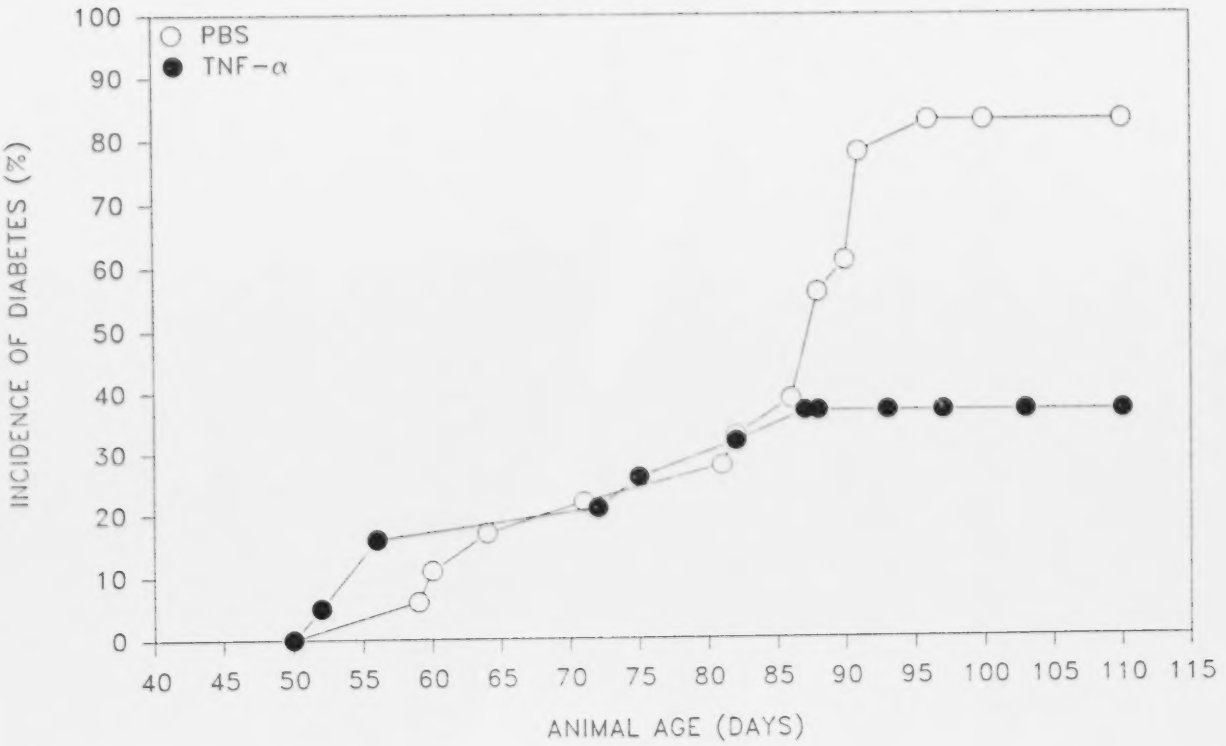

FIGURE 8.3
Effect of Tumour Necrosis Factor-α on Diabetes Development in the Diabetes-Prone BB Rat. Diabetes-prone BB rats were treated chronically 3 times a week with 20 ug recombinant TNF-α or PBS from 50 days of age to 110 days of age as described in *Materials and Methods*. Results are shown for 18 TNF-α-treated and 19 PBS-treated rats in one experiment. Statistical significance was determined by the Fisher Exact Test.

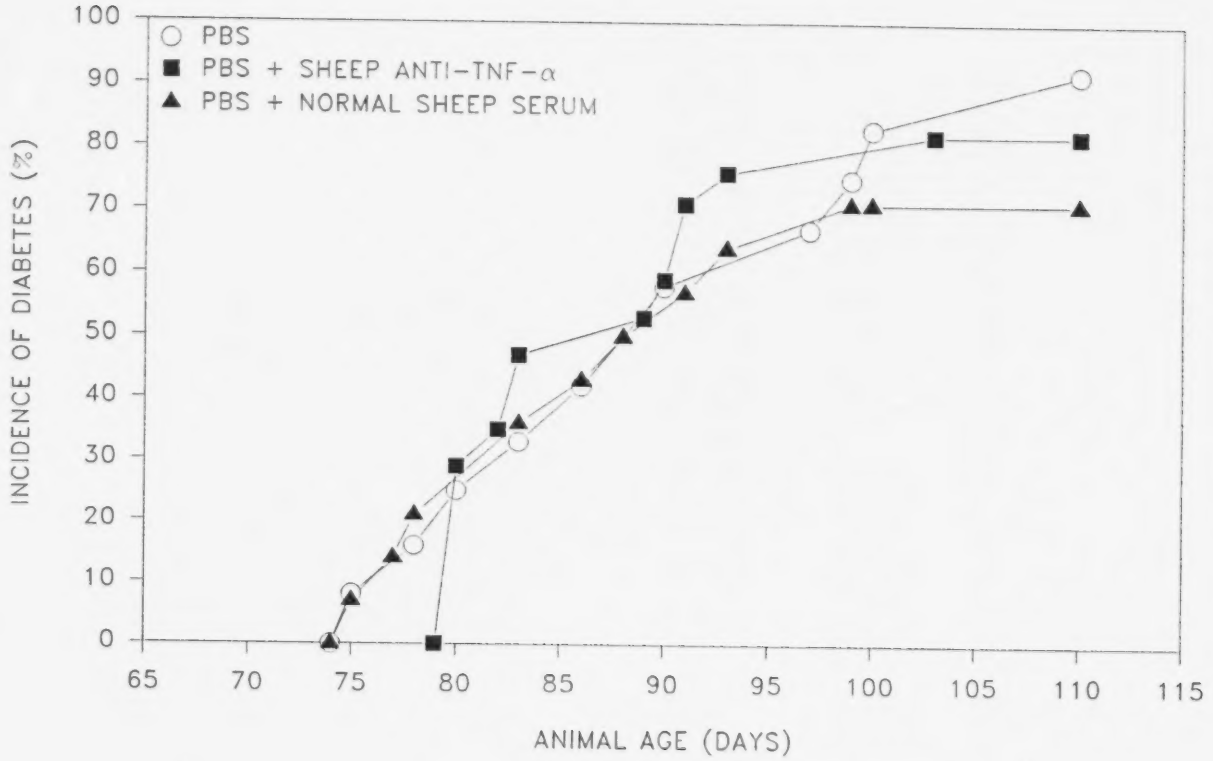

FIGURE 8.4
Effect of Sheep Antiserum to Murine TNF on Diabetes-Prone BB Rats. Diabetes-prone BB rats were treated chronically with PBS, sheep anti-murine TNF antiserum, or control sera 3 times a week from 81 days of age to 110 days of age as described in *Materials and Methods*. Results are shown for 12 PBS-treated, 14 sheep anti-murine TNF-α-antiserum-treated, and 17 control serum-treated rats in one experiment. Statistical significance was determined by the Fisher Exact Test.

CHAPTER 9

DEFICIENT TUMOUR NECROSIS FACTOR PRODUCTION BY PERITONEAL CELLS FROM DIABETES-PRONE BB RATS IS NOT DUE TO THE ABSENCE OF LIPOPOLYSACCHARIDE RECEPTORS

INTRODUCTION

In chapter 3, it was shown that tumour necrosis factor (TNF) production by diabetes-prone (DP) BB rat peritoneal cells was deficient after stimulation *in vitro* and *in vivo* with lipopolysaccharide (LPS), interferon gamma (IFN-γ), or the combination compared to diabetes-resistant (DR) BB rats. In those experiments it was also shown that peritoneal cells from DP rats which were treated with a single intraperitoneal injection of complete Freund's adjuvant (CFA), had TNF levels that were similar to peritoneal cells from DR and DR-CFA rats.

Lipopolysaccharide (LPS) activates macrophages for cytotoxic activity and cytokine production including TNF-α (393) by binding to, and activating different lipopolysaccharide receptors (LPS-R). These include receptors of 40 kDa, 70-80 kDa, CD11/18 and scavenger receptors (394). In addition, a 53 kDa glycosyl-phosphatidylinositol anchored membrane protein, CD14 is important in LPS-induced TNF-α production (395). However, LPS binding to CD14 requires prior association with a 60 kDa serum protein called lipopolysaccharide binding protein (LBP) (396, 397). The LPS-LBP complex binds to CD14 to mediate macrophage activation and TNF-α production. Because TNF production is severely reduced in DP rat peritoneal cells compared to DR rats in response to LPS, this suggests there may be a deficiency in the numbers of, or defects in LPS-R or CD14 on these cells.

Deficient production of TNF in DP rat peritoneal cells may be due to deficient numbers of, or defects in, cell surface receptors such as LPS-R and CD14. Complete Freund's adjuvant may correct these deficiencies or defects resulting in increased TNF production. This hypothesis was tested by the following strategy. Peritoneal cells from DP and DR rats were double-stained for the peritoneal macrophage marker, OX-43 and for lipopolysaccharide receptors (LPS-R) including CD14 to determine whether a deficiency in surface expression of LPS-R and/or CD14 correlates with deficient TNF production. Tumour necrosis factor levels were greater by peritoneal cells from old non-diabetic DP rats compared to young DP rats and were included in this study for comparison.

RESULTS

Flow Cytometric Analysis of Peritoneal Macrophages from BB Rats

Relative numbers of leukocytes and macrophages from the peritoneum of the different BB rat groups were determined by two colour flow cytometry. The total number of leukocytes as measured by OX-1 expression obtained by peritoneal lavage from the different BB rat groups ranged from 83 to 97%. Total numbers of OX-43$^+$ macrophages from the different rat groups ranged from 62 to 77% except for those of old DP rats which were 52% (**TABLE 9.1**). Representative 2 colour dot-plots of unstained, second antibody-stained and leukocyte-positive (OX-1$^+$)/macrophage-positive (OX-43$^+$) cells are found in **Figures 9.1**, **9.2**, and **9.3**, respectively.

Lipopolysaccharide Receptor Expression on Peritoneal Cells From BB Rats

Release of TNF in response to LPS or IFN-γ + LPS is significantly lower in DP rat peritoneal cells compared to DR rats suggesting that a deficiency in cell surface expression of lipopolysaccharide receptor expression may exist. Peritoneal cells were stained for both lipopolysaccharide receptor (LPS-R) or CD14 cell surface expression by an indirect method using fluorescein isothiocyanate-conjugated (FITC)-LPS. Total (single positive and double positive) LPS-R$^+$ cells ranged from 80 to 90% and were similar in all rat groups (**TABLE 9.2**). Complete Freund's adjuvant treatment significantly increased the percentages of LPS-R$^+$ OX-43$^-$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.005$) rats, and DP-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.05$) DP rats. However, CFA treatment significant decreased the percentages of LPS-R$^+$ OX-43$^+$ macrophages from both DR-CFA rats compared to young and old ($P \leq 0.001$) DR rats, and DP-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.005$) DP rats. This decrease was not due to losses in peritoneal cell macrophage numbers, but due to a loss of OX-43 cell surface expression on cells obtained from CFA-treated rats. The number of macrophages are similar in these populations (**TABLE 9.1**). A representative two-colour dot-plot for LPS-R$^+$/OX-43$^+$ cells is presented in **Figure 9.4**.

Complete Freund's adjuvant significantly increased the percentages of total (single positive and double positive) CD14$^+$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.001$) DR rats (**TABLE 9.3**). Complete Freund's adjuvant treatment significantly increased the percentages of total (single positive and double positive) CD14$^+$ peritoneal cells from DP-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.05$) DP rats. Similar to LPS-R$^+$ cells, CFA treatment of DR and DP rats significantly decreased the expression of OX-43 on macrophages expressing CD14 (CD14$^+$ OX-43$^+$) compared to young ($P \leq 0.001$) and old ($P \leq 0.001$) DR rats, and young ($P \leq 0.001$) DP rats, respectively. Again, this is not due to a loss in the numbers of macrophages (**TABLE 9.1**) but due to the loss of OX-43 cell surface expression on these cells. A representative two-colour dot-plot CD14$^+$/OX-43$^+$ cells is presented in **Figure 9.5**.

Fluorescence intensity (FL1) of LPS-R and CD-14 expressing cells were analyzed from single histograms and two-colour dot plots (**TABLE 9.4**). Complete Freund's adjuvant significantly decreased the fluorescence intensity (FL1) in LPS-R$^+$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.05$) and old ($P \leq 0.01$) DR rats, and Xmean (—/+) compared to old DR rats ($P \leq 0.005$). Complete Freund's adjuvant significantly decreased the fluorescence intensity (FL1) in LPS-R$^+$ peritoneal cells from DP-CFA rats compared to young ($P \leq 0.01$) and old ($P \leq 0.05$) DP rats, Xmean (+/+) compared to young ($P \leq 0.05$) and old ($P \leq 0.001$) DP rats, and Xmean (—/+) compared to young DP rats ($P \leq 0.05$). In contrast, CFA treatment significantly increased the Xmean (+/+) intensity in CD14$^+$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.001$) and old ($P \leq 0.005$) DR rats, and fluorescence intensity (FL1) in CD14$^+$ peritoneal cells from DP-CFA rats compared to old DP rats ($P \leq 0.05$), and Xmean compared to young DP rats ($P \leq 0.01$).

Peak channel fluorescence of LPS-R and CD-14 expressing peritoneal cells were analyzed from single histograms and two-colour dot plots (**TABLE 9.5**). Complete Freund's adjuvant significantly decreased the peak channel fluorescence in LPS-R$^+$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.05$) and old ($P \leq 0.001$) DR rats, and in LPS-R$^+$ peritoneal cells from DP-CFA rats compared to young ($P \leq 0.05$) and old ($P \leq 0.05$) DP

rats. In contrast, CFA treatment significantly increased the peak channel fluorescence of $CD14^+$ peritoneal cells from DR-CFA rats compared to young ($P \leq 0.005$) and old ($P \leq 0.005$) DR rats. No significant differences were observed in $CD14^+$ peritoneal cells from DP-CFA rats compared to pertioneal cells from young and old DP rats.

DISCUSSION

Deficient TNF production is implicated in the onset of insulin-dependent diabetes mellitus. In Chapter 3, it was shown that TNF production by DP rat peritoneal cells is deficient after stimulation *in vitro* or after stimulation *in vivo* with IFN-γ, with LPS, or the combination. It was also shown that peritoneal cells obtained from CFA-treated DP rats had released increased amounts of TNF after stimulation *in vitro*. These preliminary findings were extended in this chapter to determine whether the deficient TNF production by these pertioneal cells correlates with deficient numbers of LPS-R and/or CD14.

Deficient TNF production by DP rat peritoneal cells does not correlate with lower numbers of peritoneal leukocytes or more importantly, peritoneal macrophages. In previous experiments, priming of DP rat peritoneal cells with IFN-γ concurrently with LPS does not correct deficient TNF production when compared to stimulation with LPS alone had suggested that there may be deficient peritoneal cell LPS-R and/or CD14 cell surface expression. As shown in this chapter, LPS-R or CD14 expression on peritoneal cells and macrophages is not deficient. Complete Freund's adjuvant treatment increased the numbers of cells expressing LPS-R and CD14 molecules, and increased the number of receptors/cell in the macrophage population, at least with CD14. The first conclusion that can be drawn from these experiments is that deficient TNF production by DP rat peritoneal macrophages is not due to a decreased numbers of cells expressing LPS-R or CD14 nor is it due to a deficiency in the number of receptors expressed on the surface of these cells. However, this does not rule out the possibility that these receptors may be defective in transducing a signal necessary for activation of the macrophage and production of TNF. These results also suggest indirectly, that TNF production may be suppressed by an unidentified factor(s).

Unexpectedly, it was shown that the peritoneal macrophage cell surface marker, OX-43, was greatly reduced on peritoneal macrophages from CFA-treated DR and DP rats. The loss of this cell surface marker occurred as a result of culturing CFA-treated DP and DR peritoneal cells in the presence of LPS. No loss of cell surface expression of OX-43 occurred with peritoneal cells from PBS-treated or untreated DR and DP rats. This is the first report of the loss of OX-43. No information on the function of OX-43 has been reported except that it is a heat-resistant 90 kDa protein that is not decreased on activated or 4-day thioglycollate-stimulated peritoneal cells. Furthermore, it does not seem to play any role in macrophage phagocytosis (360). It is plausible that OX-43 may be a differentiation/maturation marker specific to macrophages in the peritoneal cavity, and that an additional effect of CFA may be to induce further differentiation/maturation of the peritoneal macrophage population and hence increase TNF production in response to stimuli. This does seem plausible since increases in TNF production may act in an autocrine manner resulting in macrophage differentiation and/or maturation (321). Further support for the actions of CFA as a differentiation/maturation factor in DP rat peritoneal macrophages is the increase in CD14 expression. CD14 expression can be up-regulated in immature macrophage cell lines in response to PMA, IFN-γ, or vitamin D3. Treatment

with TNF-α or LPS also induces CD14 (398). Since PMA, IFN-γ, vitamin D3 and LPS are known to stimulate TNF production, it is plausible that CFA, through the action of increased TNF production results in increased CD14 expression. Maturation differences between untreated and CFA-treated DP rat peritoneal macrophages is also suggested by the inability of the former to adhere to fetal bovine serum-tissue culture-treated plastic dishes whereas peritoneal macrophages from CFA-treated DP rats do (personal observation). OX-43 does not appear to be involved in TNF production since unmanipulated peritoneal macrophages from DP rats stained for both the OX-43 and LPS-R/CD14. A more detailed study into BB rat macrophage maturation and/or differentiation is suggested and may be a possible avenue for future research.

SUMMARY

Deficient TNF production is not related to deficient numbers of LPS-R and CD14 since peritoneal cells from untreated and CFA-treated DP rats express these receptors. However, there is an apparent increase in the number of pertioneal cells which express these receptors, and an increase in the number of receptors per cell from those animals which were CFA-treated. Furthermore, the novel observation, that of the loss of the OX-43 marker on CFA-treated macrophages suggests that CFA could participate, albeit, indirectly, with macrophage maturation or differentiation to become TNF production competent. These results suggest that deficient TNF production by DP rat peritoneal cells is not due to deficiencies in LPS-R and CD14 expression, but rather may be due to a generalized suppression of cytokine production. Future experiments could be done to determine whether generalized suppression of cytokine production occurs in DP rat peritoneal cells. These experiments would include the addition of pharmacological agents or neutralizing antibodies to known suppressor molecules such as members of the prostaglandin family, or cytokines such as interleukin 4, interleukin 10, and interleukin 13 and measuring TNF levels after stimulation *in vitro* with IFN-γ, LPS or the combination.

TABLE 9.1

FLOW CYTOMETRIC DETERMINATION OF PERITONEAL CELLS FROM BB RATS

BB rat group	Percent Positive Cells		
	OX-1 TTL	OX-1$^+$ OX-43$^-$	OX-1$^+$ OX-43$^+$
DR	90.0 ± 0.5	16.7 ± 0.6	73.3 ± 0.8
DP	86.1 ± 0.8	13.4 ± 1.3	72.7 ± 1.2
DR-OLD	82.6 ± 3.8	13.8 ± 1.6	68.8 ± 4.6
DP-OLD	86.2 ± 2.0	33.9 ± 4.1	52.2 ± 4.2
DR-CFA	96.6 ± 0.3	35.0 ± 2.1	61.9 ± 2.2
DP-CFA	93.6 ± 1.2	16.3 ± 2.2	77.3 ± 2.8

NOTE: Values are Means ± S.E. for percent positive cells from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

TABLE 9.2

TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF LIPOPOLYSACCHARIDE RECEPTOR (LPS-R)-POSITIVE PERITONEAL CELLS FROM BB RATS

BB rat group	Percent Positive Cells			
	TTL LPS-R$^+$	LPS-R$^+$ OX-43$^-$	LPS-R$^+$ OX-43$^+$	TTL OX-43$^+$
DR	83.5 ± 1.5	33.3 ± 1.0	50.1 ± 1.4	50.1 ± 1.4
DP	89.2 ± 1.0	33.2 ± 2.4	56.0 ± 2.9	56.0 ± 2.9
DR-OLD	81.7 ± 4.7	31.0 ± 1.9	50.7 ± 5.3	50.7 ± 5.3
DP-OLD	89.2 ± 4.0	69.4 ± 2.1	19.9 ± 5.3	19.9 ± 5.3
DR-CFA	79.9 ± 1.0	70.4 ± 1.1^{*,C}	9.5 ± 0.2^{*,B}	9.5 ± 0.2^{*,B}
DP-CFA	90.1 ± 1.3	63.8 ± 4.7^{*,A}	26.4 ± 3.5^{*,C}	26.4 ± 3.5^{*,C}

NOTE: Values are Means ± S.E. for percent positive cells from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^*P \leq 0.001$ compares DR-CFA to DR and DP-CFA to DP.
$^AP \leq 0.05$, $^BP \leq 0.005$, $^CP \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 9.3

TWO-COLOUR FLOW CYTOMETRIC DETERMINATION OF LIPOPOLYSACCHARIDE-RECEPTOR CD14-POSITIVE PERITONEAL CELLS FROM BB RATS

BB rat group	Percent Positive Cells				
	TTL $CD14^+$	$CD14^+$ $OX\text{-}43^-$	$CD14^+$ $OX\text{-}43^+$	$CD14^-$ $OX\text{-}43^+$	TTL $OX\text{-}43^+$
DR	55.8 ± 3.2	11.6 ± 1.1	44.2 ± 2.5	6.8 ± 1.9	51.0 ± 1.7
DP	65.7 ± 1.8	12.1 ± 1.4	53.6 ± 2.5	6.5 ± 1.3	60.1 ± 2.6
DR-OLD	50.9 ± 2.6	9.2 ± 1.2	41.7 ± 2.3	12.2 ± 3.8	53.9 ± 4.6
DP-OLD	54.4 ± 9.0	33.6 ± 4.7	20.8 ± 5.2	0.2 ± 0.2	21.0 ± 5.2
DR-CFA	55.2 ± 1.6	50.1 ± 1.4^{*,B}	5.1 ± 0.2^{*,B}	0.00	5.1 ± 0.2^{*,B}
DP-CFA	72.2 ± 1.1	52.7 ± 3.7^{*,A}	19.5 ± 2.8*	0.00	19.5 ± 2.8*

NOTE: Values are Means ± S.E. values for percent positive cells from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^{*}P \leq 0.001$ compares DR-CFA to DR and DP-CFA to DP.
$^{A}P \leq 0.05$, $^{B}P \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 9.4

FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR THE LIPOPOLYSACCHARIDE RECEPTOR (LPS-R) AND CD14

BB rat group	Fluorescence Intensity					
	LPS-R			CD14		
	FL1	Xmean (+/+)	Xmean (—/+)	FL1	Xmean (+/+)	Xmean (—/+)
DR	149.3 ± 17.0	119.8 ± 14.3	39.3 ± 3.9	32.8 ± 3.2	21.4 ± 1.3	20.1 ± 1.9
DP	214.3 ± 22.9	141.2 ± 15.8	53.4 ± 7.2	35.7 ± 4.0	22.3 ± 1.5	20.8 ± 2.7
DR-OLD	183.7 ± 22.4	134.8 ± 13.1	41.9 ± 2.8	37.1 ± 6.3	21.5 ± 2.0	23.6 ± 2.8
DP-OLD	213.2 ± 31.1	272.3 ± 20.7	70.2 ± 9.6	79.8 ± 13.1	51.0 ± 6.6	32.9 ± 6.4
DR-CFA	64.4 ± 3.6^{*,B}	129.3 ± 11.5	25.6 ± 0.3^{C}	41.4 ± 5.7	68.1 ± 11.2^{***,C}	24.2 ± 1.4
DP-CFA	88.1 ± 4.3^{**,A}	79.0 ± 3.8^{*,D}	31.3 ± 1.1^{A}	32.5 ± 3.5^{A}	35.2 ± 4.6**	16.7 ± 1.2

NOTE: Values are Means ± S.E. for fluorescence channels. Abbreviations used indicate mean channel fluorescence for single histogram analysis (FL1) and two-colour dot plot position (Xmean; where +/+ represents double positive cells and —/+ represents single positive cells for LPS-R or CD14) analysis. Cells were obtained from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compares DR-CFA to DR and DP-CFA to DP.
$^{A}P \leq 0.05$, $^{B}P \leq 0.01$, $^{C}P \leq 0.005$, $^{D}P \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 9.5

PEAK CHANNEL FLUORESCENCE OF LIPOPOLYSACCHARIDE RECEPTOR (LPS-R) AND CD14 ON BB RAT PERITONEAL CELLS

BB rat	Peak Channel Fluorescence	
group	LPS-R	CD14
DR	71.7 ± 7.9	12.8 ± 1.9
DP	65.8 ± 7.5	12.8 ± 1.6
DR-OLD	63.2 ± 2.4	10.1 ± 1.4
DP-OLD	54.7 ± 6.9	18.5 ± 5.9
DR-CFA	30.4 ± 2.2$^{*, C}$	27.8 ± 3.1$^{**, B}$
DP-CFA	33.9 ± 0.9$^{*, A}$	18.4 ± 5.0

NOTE: Values are Means ± S.E. for peak channel fluorescence. Values were obtained from single colour histograms.

$^{*}P \leq 0.05$, $^{**}P \leq 0.005$ compares DR-CFA to DR and DP-CFA to DP.
$^{A}P \leq 0.05$, $^{B}P \leq 0.005$, $^{C}P \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

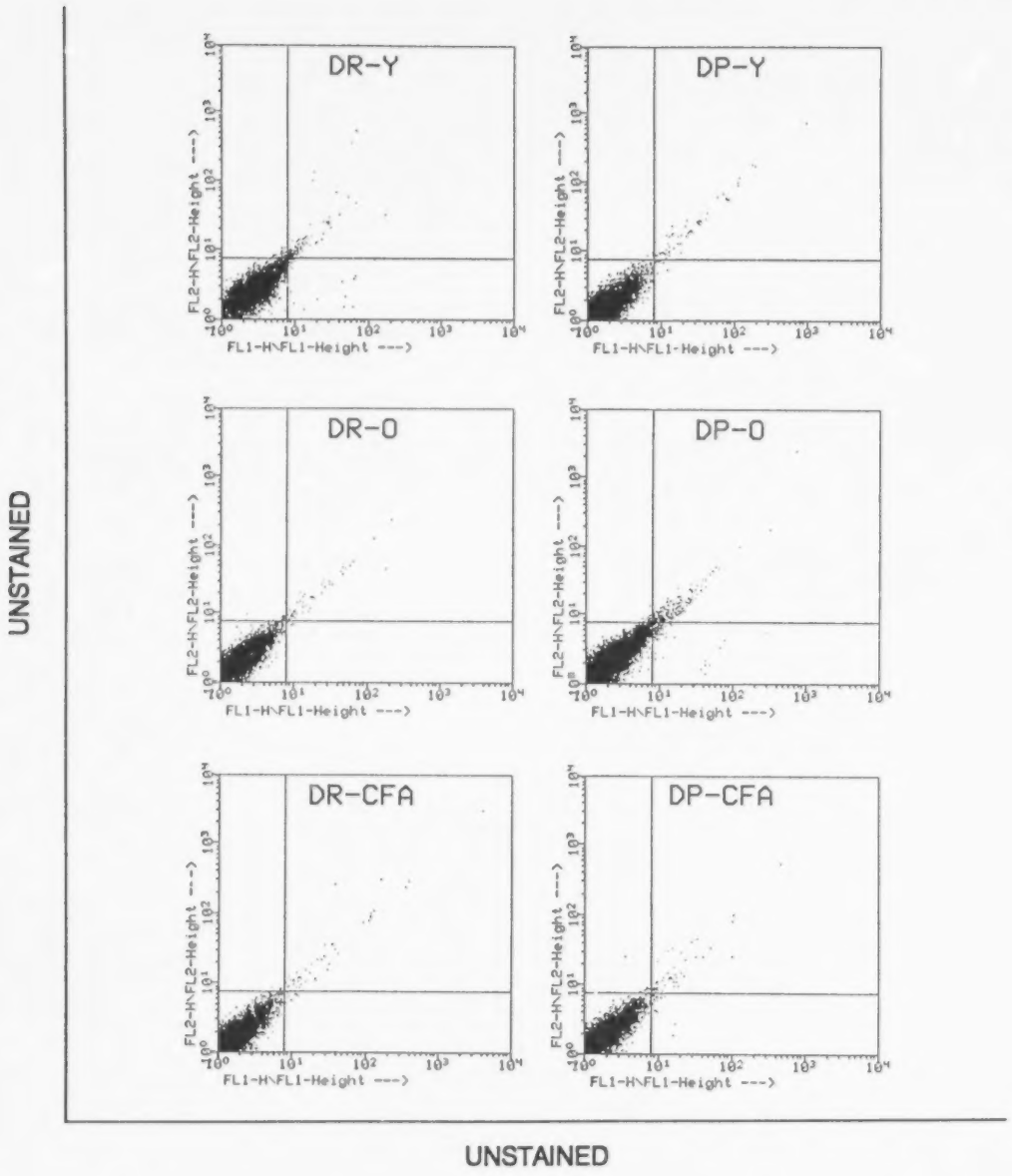

FIGURE 9.1
Representative two-colour dot-plots of unstained peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling. Positive cells are considered autofluorescent.

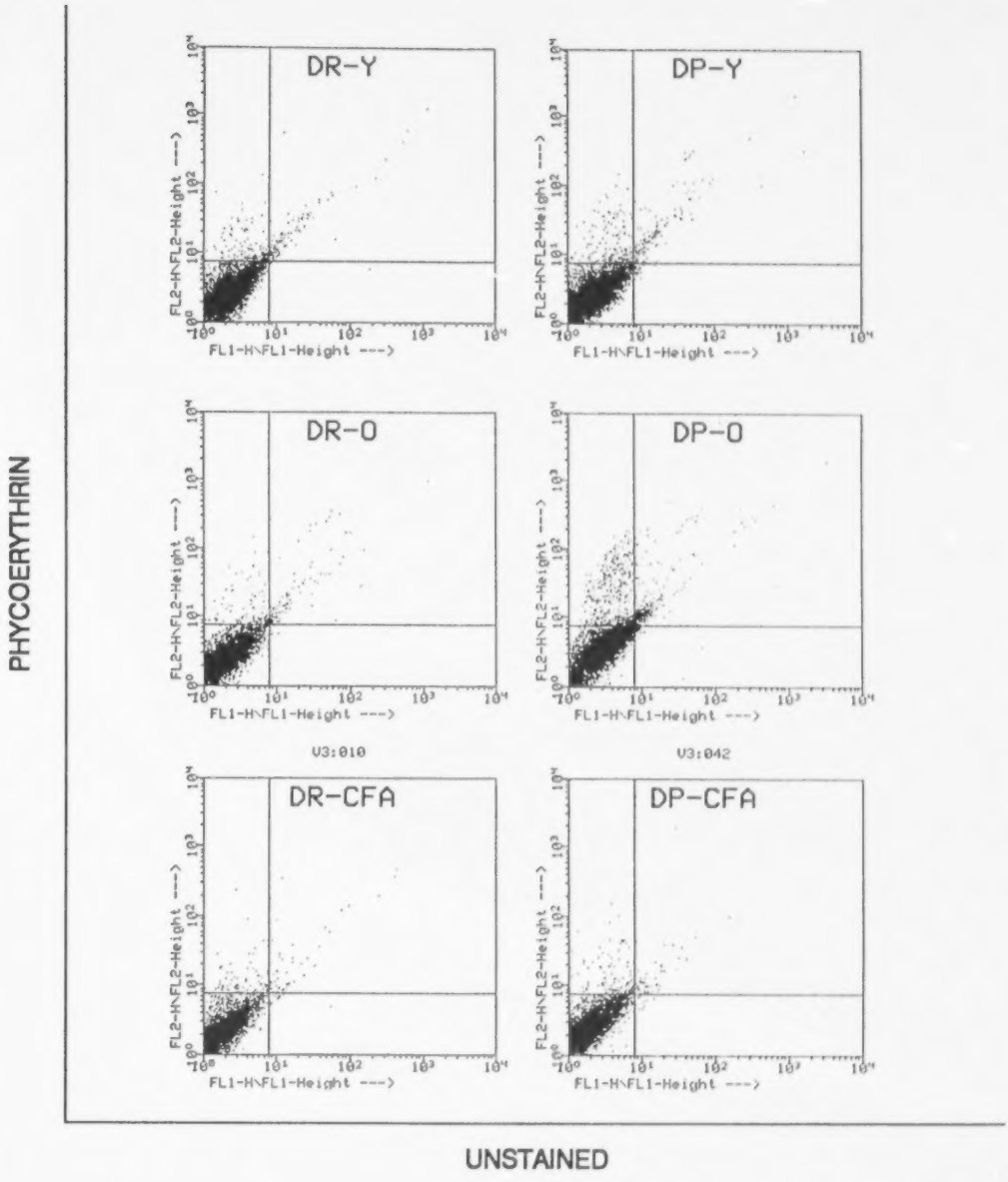

FIGURE 9.2
Representative two-colour dot-plots of control second antibody-labelled peritoneal cells young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells remained unlabelled for FL1 and were labelled with a goat anti-mouse $F(ab')_2$-PE (phycoerythrin) antibody (FL2). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive.

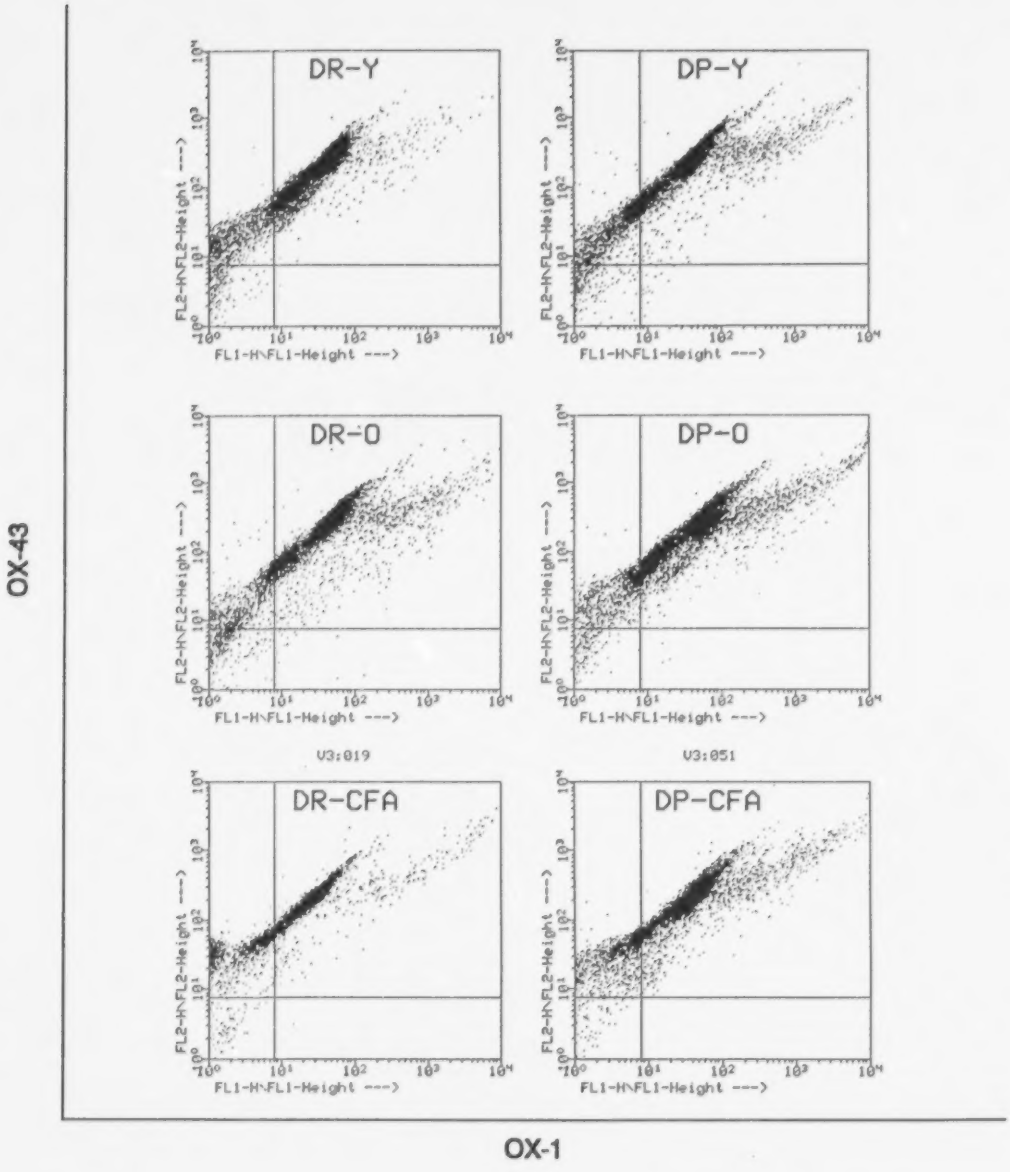

FIGURE 9.3
Representative two-colour dot-plots of antibody-labelled peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with OX-1 + goat anti-mouse-F(ab')$_2$-PE (FL2) and OX-43-FITC (FL1). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive.

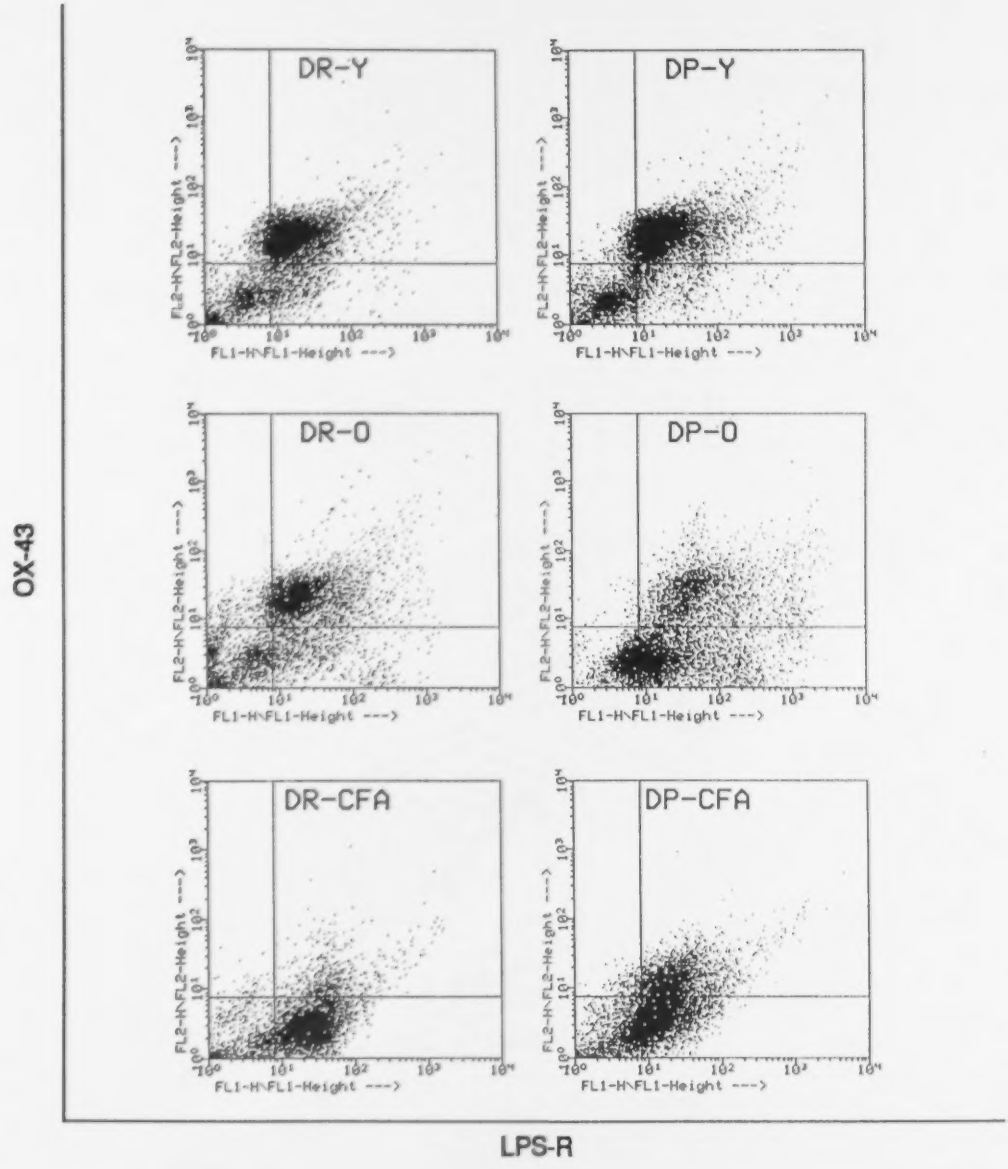

FIGURE 9.4
Representative two-colour dot-plots of antibody-labelled peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with FITC-conjugated LPS in PBS (FL1) and OX-43 + goat anti-mouse $F(ab')_2$-PE (FL2). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling.

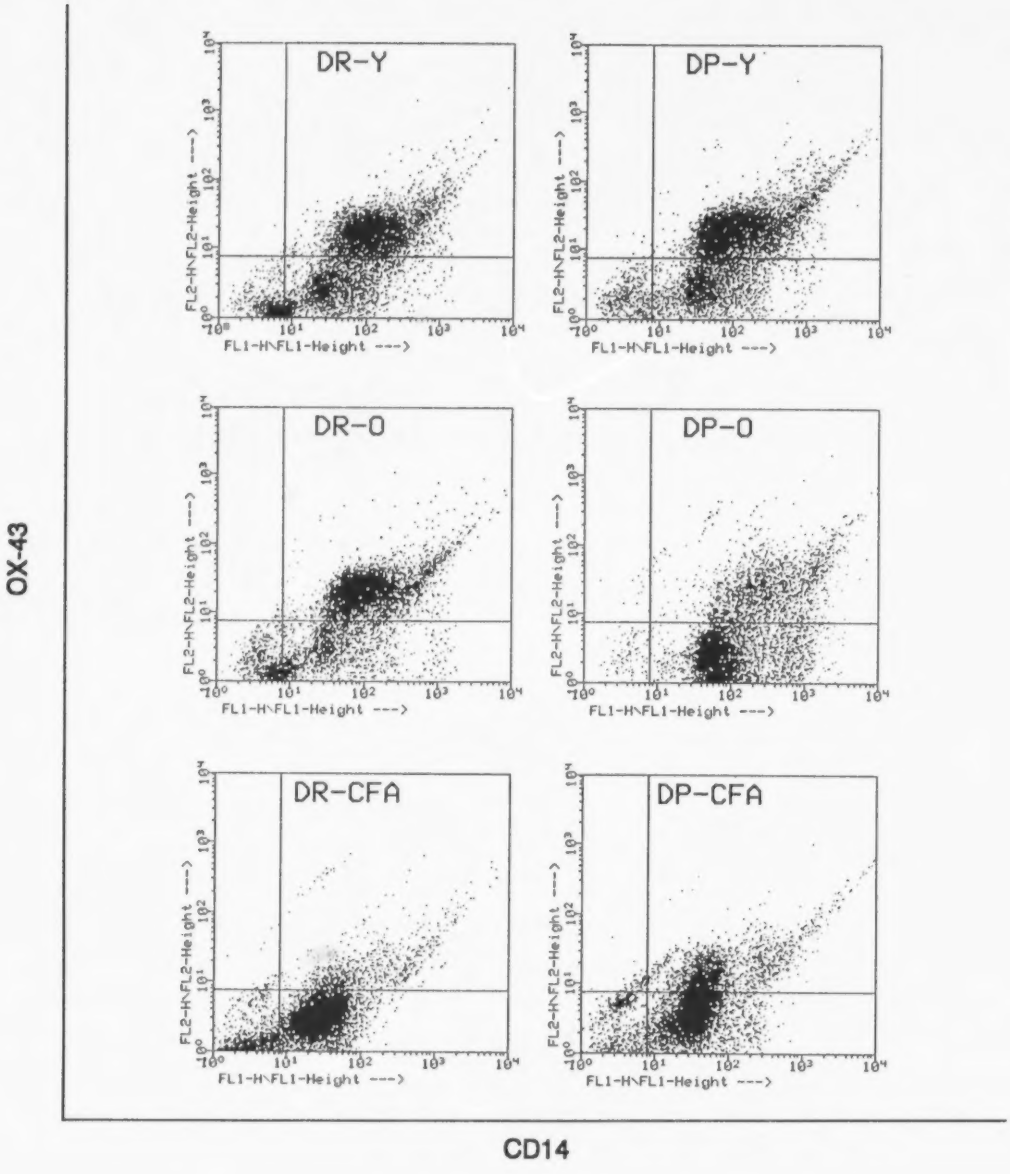

FIGURE 9.5
Representative two-colour dot-plots of antibody-labelled peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with FITC-conjugated LPS in 10% goat serum (FL1) and OX-43 + goat anti-mouse $F(ab')_2$-PE (FL2). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling.

CHAPTER 10

ALTERNATE MECHANISMS OF MACROPHAGE ACTIVATION FAIL TO STIMULATE DIABETES-PRONE BB RAT PERITONEAL CELL TUMOUR NECROSIS FACTOR PRODUCTION: CORRECTION BY INDOMETHACIN OR COMPLETE FREUND'S ADJUVANT

INTRODUCTION

Activation of macrophages and release of tumour necrosis factor (TNF) can be mediated by bacterial products such as lipopolysaccharide (LPS)(393), cytokines such as interleukin 2 (IL-2) and interferon gamma (IFN-γ)(399, 400), activators of protein kinase such as phorbol, 12-myristate, 13-acetate (PMA), and calcium channel agonists such as A23187 (401). Lipopolysaccharide activates macrophages and TNF production by receptor-dependent mechanisms involving protein kinases (401), while IFN-γ acts through its receptor to activate Ca^{2+} mobilization and calmodulin-dependent protein kinases (402). In contrast, TNF-α production can be suppressed by lymphocyte and macrophage cytokines such as interleukin 4 (IL-4)(403), interleukin 10 (IL-10)(404), interleukin 13 (IL-13)(405), and members of the prostaglandin family including PGE_2 (406). Cytokines can regulate macrophage activation and cytokine production, and more importantly, these cells can autoregulate their own cytokine production through IL-10, IL-13 and PGE_2.

As shown in chapter 3, TNF production by diabetes-prone (DP) BB rat peritoneal cells is deficient in response to stimuli *in vivo* or *in vitro*. In chapter 9, it was shown that levels of LPS-R and CD14 were not deficient on these cells, thereby excluding deficiencies in LPS-R numbers as a cause for defective TNF production. However, this does not exclude the possibility of defects in signal transduction mechanisms through different LPS-R or through the IFN-γ receptor. There is the possibility that no defects exists in receptor activation of signal transduction mechanisms but rather, TNF production by DP rat peritoneal cells is suppressed by cytokines or prostaglandins. Furthermore, it is not known whether DP rat peritoneal cell TNF production is also deficient in response to other agents.

Deficient TNF production can be corrected in those DP rats which were CFA-treated and these levels are similar to diabetes-resistant (DR) and DP rats which never developed diabetes (DP-OLD). However, it is not known by which mechanism(s) CFA corrects this deficiency. The hypothesis that defects in receptor-mediated signal transduction mechanisms or suppression by cytokines or prostaglandins may be responsible for deficient TNF production by DP rat peritoneal cells and correction of these defects or suppression was tested. The strategies used to test the hypothesis involved repeating earlier experiments in which DP rat peritoneal cells were stimulated with IFN-γ and LPS, and extended in experiments whereby T_{h1} cytokines, IFN-γ and/or IL-2, and protein kinase and/or calcium channel agonists were used to determine whether normal TNF production was stimulated or whether defects in signal transduction pathways leading to TNF may responsible for this deficiency. Finally, experiments were done to determine whether another macrophage product, prostaglandin E_2 was responsible for suppressing TNF production and whether non-specific immunostimulation down-modulates PGE_2.

RESULTS

TNF Production after Stimulation In Vitro

Peritoneal cells were incubated *in vitro* in control medium, in medium containing IFN-γ, LPS or in medium containing IFN-γ + LPS (for concentrations, see legend) (**FIGURE 10.1**). Under these conditions, release of TNF (pg/5 X 10^5 cells) was significantly lower for DP than DR peritoneal cells incubated in control medium ($P \leq 0.01$), in medium containing LPS ($P \leq 0.001$ for DR *vs.* DP and $P \leq 0.005$ for DR-OLD *vs.* DP-OLD), and in medium containing IFN-γ + LPS ($P \leq 0.01$ for DR *vs.* DP and $P \leq 0.05$ for DR-OLD *vs.* DP-OLD). These findings are similar to those presented earlier.

Release of Tumour Necrosis Factor Remains Deficient in Response to Helper T_1 Lymphocyte Proinflammatory Cytokines

Deficient release of DP rat peritoneal cell TNF in response to LPS or IFN-γ + LPS is not due to decreased expression of LPS-R and/or CD14, suggesting that a defect(s) may be associated with the receptor itself or in signal transduction pathways associated with these receptors. Proinflammatory cytokines from helper T_1 lymphocytes including IL-2 and IFN-γ are capable of stimulating TNF production by peritoneal cells. These cytokines were used in combination together to stimulate TNF production in DP rat peritoneal cells. Results are shown in **Figure 10.2**. Peritoneal cells from DP rats stimulated with IL-2 released significantly less TNF compared to DR rat peritoneal cells ($P \leq 0.005$ for DP *vs.* DR and $P \leq 0.01$ for DP-OLD *vs.* DR-OLD), with IL-2 + IFN-γ ($P \leq 0.05$ for DP *vs.* DR and $P \leq 0.005$ for DP-OLD *vs.* DR-OLD), and LPS + IL-2 ($P \leq 0.001$ for DP *vs.* DR and $P \leq 0.01$ for DP-OLD *vs.* DR-OLD).

Receptor-Independent Activation of Peritoneal Cells by Phorbol, 12-Myristate, 13-Acetate and Calcium Channel Agonists Fail to Increase DP Rat TNF Production

The above experiments indicating that TNF production by DP peritoneal cells remains deficient after treatment with T_{h1} lymphocyte proinflammatory cytokines, IL-2 and IFN-γ, and IL-2 + LPS suggests that a defect may be present in signal transduction pathways rather than with the LPS-R or CD14 receptors. Two known signal transduction pathways, protein kinases and Ca^{2+}-stimulated calmodulin-dependent protein kinases were activated directly with phorbol, 12-myristate, 13-acetate (PMA), A23187, or PMA + A23187 (**Figure 10.3**). Peritoneal cells from DP rats produced significantly less TNF compared to DR rat peritoneal cells in response to PMA ($P \leq 0.05$ for DR *vs.* DP), to A23187 ($P \leq 0.001$ for DR *vs.* DP and $P \leq 0.01$ for DR-OLD *vs.* DP-OLD) and to PMA + A23187 ($P \leq 0.001$ for DR *vs.* DP and DR-OLD *vs.* DP-OLD). These results suggest that there are no defects in these two signal transduction pathways leading to deficient TNF production, but rather, TNF production by DP rat peritoneal cells may be suppressed by other cytokines or prostaglandins.

Stimulation with the Combination of Lipopolysaccharide and Indomethacin Eliminates the Differences in Tumour Necrosis Factor Production by Peritoneal Cells from DP and DR Rats

The above results indicate that receptor co-stimulation with T_{h1} cytokines nor direct stimulation of two signal transduction pathways eliminates the differences in TNF production by DR and DP peritoneal cells. These results do suggest, however, that there may be a generalized suppression of cytokine production in DP rat macrophages and that this suppression can be overcome after treatment with CFA.

Peritoneal cells from DP and DR rats that had been treated with saline or CFA 10-14 days earlier were collected and then cultured in medium alone, in medium containing LPS, in medium containing indomethacin (INDO), or in medium containing LPS + INDO (see legend for concentrations). Released TNF was measured after 6 hours of *in vitro* culture (**Figure 10.4**). Released TNF by LPS-stimulated DP peritoneal cells was significantly less than when LPS + INDO stimulated ($P \leq 0.05$) and is similar to peritoneal cells from DP-CFA rats cultured with LPS alone or LPS + INDO. TNF release by peritoneal cells from DP and DP-CFA rats were similar after culture with INDO alone.

Addition of indomethacin to DP rat peritoneal cells cultured with LPS had TNF levels comparable to those observed from CFA-treated DP rat peritoneal cells, suggesting that prostaglandins may be responsible for the deficiency (suppression) in TNF production observed. In this experiment, unstimulated release of TNF by PBS-treated DR rat peritoneal cells were greater than observed in earlier experiments, and usually similar to those levels observed by PBS-treated DP rat peritoneal cells. As a result, TNF production by CFA-treated DR rat peritoneal cells in response to all stimuli appear reduced.

Levels of prostaglandin E_2 (PGE_2) were determined in samples of the same media used to determine released TNF (**Figure 10.5**). Prostaglandin E_2 levels were significantly reduced in media from peritoneal cells from DR-CFA or DP-CFA rats after stimulation with LPS when compared to DR-PBS ($P \leq 0.01$) and DP-PBS ($P \leq 0.001$) rats, respectively. Prostaglandin E_2 levels were significantly decreased in all rat groups after culture in media containing LPS + INDO when compared to culture in media containing LPS only ($P \leq 0.005$ for DR; $P \leq 0.01$ for DR-CFA; $P \leq 0.001$ for DP-PBS; and $P \leq 0.005$ for DP-CFA). These results suggest that TNF production by DP rat peritoneal cells suppressed by over-production of PGE_2 or hyper-sensitivity to PGE_2.

DISCUSSION

The results above show that TNF production by DP rat peritoneal cells remains deficient after stimulation with T_{h1} cytokines, IFN-γ and IL-2, in combination with each other and in combination with LPS. This further suggests that deficient TNF production may be due to defects in receptor-mediated signal transduction pathways. To address this, DP rat peritoneal cells were activated with PMA, A23187 or in combination which activate protein kinases and Ca^{2+}-stimulated calmodulin-dependent protein kinases. Since these agents activate cells directly without binding specific receptors, a general assessment of these two signal transduction pathways were done. Again, TNF production remained deficient. These results suggest that deficient TNF production by DP rat peritoneal cells is not due to either defects in transduction of signals necessary for stimulation of TNF production, but rather may be due to a generalized suppression by some other factor. Factors known to down-regulate or suppress TNF production include IL-4 (403), IL-10 (404), IL-13 (405) and PGE_2 (406). From these experiments, it is not possible to rule out IL-4 even though cells recovered in a peritoneal lavage from a healthy rat without prior stimulation or recruitment contains of about 60-70% macrophages, 20-30% neutrophils, and less than 10% lymphocytes. In addition to presence of IL-4, IL-10, IL-13 and PGE_2 may be considered since each is a macrophage product and has also been shown to suppress TNF production.

In a separate series of experiments, PBS- and CFA-treated DR and DP rat peritoneal cells were cultured in the presence of a cyclooxygenase pathway inhibitor (and thus inhibits prostaglandin synthesis), indomethacin or indomethacin + LPS. Those DP rat peritoneal cells which were cultured in the presence of indomethacin alone or with LPS had increased levels of TNF production. Peritoneal cells from CFA-treated DP rats cultured with LPS alone resulted in levels of TNF similar to peritoneal cells from PBS-treated DP rats which were cultured with indomethacin + LPS. Taken together, these results indicate that TNF production by DP rat peritoneal cells may be suppressed by PGE_2 and is not due to defects in signal transduction pathways. These results also suggest that CFA may act by reducing PGE_2 production resulting in increased TNF production.

Prostaglandin E_2 levels were measured in the same media that TNF was measured to verify that PGE_2 may be responsible for suppressing TNF production by DP rat peritoneal cells. Two findings are clear, the first is that treatment of DP rats with CFA alters the state of the macrophages and thus suppresses unstimulated PGE_2 production. Secondly, in response to LPS, peritoneal cells from CFA-treated DP rats produce significantly less PGE_2 than their PBS controls. Overall, these results provide evidence that TNF production by DP rat peritoneal cells may be suppressed by over-production of PGE_2, at least in response to LPS. However, similar levels of PGE_2 were produced by unstimulated DP and DR peritoneal cells suggesting that suppression of TNF production by DP rat peritoneal cells may be due to hyper-sensitivity of these cells to the effects of PGE_2. This does not discount the effect of CFA on PGE_2 since both CFA-treated DR and DP rat peritoneal cells produced significantly lower levels of PGE_2. Deficient TNF production by DP rat peritoneal cells may be due to suppression by other macrophages products as well, including IL-10. Overall, these results demonstrate that another mechanism by which CFA may correct deficient TNF production by peritoneal cells is by suppressing PGE_2 production or reducing DP rat peritoneal cell sensitivity to PGE_2.

This finding is contrasted to that of another group which reported an under-production of PGE_2 and an over-production of TNF by DP rat macrophages (337). However, these investigators primed these macrophages with *Corynebacterium parvum* prior to studying them *in vitro* (336). The studies undertaken here employed naive, unmanipulated macrophages prior to culture and more closely resemble the *in vivo* situation or macrophages from CFA-treated rats which do resemble those that were *Corynebacterium parvum* stimulated. BB rats have also been treated with PGE_1 in order to prevent disease. Those authors report that, in fact, diabetes was accelerated (304). This observation is in accord with our observations that low TNF levels correlate with more rapid disease onset. Taken together, these observations further suggests that threshold levels of TNF production is necessary for protection against diabetes development. This is in accord with the observation in chapter 6 in which diabetes-prone BB rats which never developed diabetes have TNF levels similar to DR rats.

A number of pathways are associated with LPS-mediated activation of macrophages and include: increases in cytosolic free Ca^{2+} (407), activation of guanine nucleotide-binding (G) protein, myristoyl transferase, phospholipase C (PLC), protein kinase C (408), and protein tyrosine kinase (409). By stimulating DP rat peritoneal macrophages with IL-2, IFN-γ, PMA, or A23187 and not returning the production of TNF to normal values, suggests that signal transduction pathways mediated by the IL-2R, IFN-γR, and more importantly LPS-R and

CD14 are intact even though TNF production is suppressed. Prostaglandin E_2 stimulates cyclic adenosine monophosphate (cAMP) accumulation (410, 411). Increases in cAMP is associated with suppression of TNF production by activating cellular phosphatases which could antagonize the net potential PKC and protein tyrosine kinase phosphorylation of proteins including NF-kB (IkB is phosphorylated allowing NF-kB to translocate to the nucleus whereby its binds to a recognition sequence on the DNA), a DNA binding protein associated with the induction of TNF production (412). Increased cellular phosphatase activity could dephosphorylate IkB, thereby inhibiting translocation of NF-kB to the nucleus, DNA binding, and TNF gene transcription. As a result, TNF production is suppressed and thus can appear to be deficient as what is observed with DP rat macrophages.

Diabetes-resistant BB rat peritoneal cells appear not to fit into this scheme based on the findings from this preliminary experiment. It will be necessary to repeat this experiment to verify the DP results and to establish PGE_2 levels for DR rat peritoneal cells. However, one explanation for the observation that DR rat peritoneal cells produce similar levels of PGE_2 compared to DP rats, yet release greater amounts of TNF, may be due to reduced numbers of PGE_2 receptors on the cell surface or DR rat peritoneal cells are less sensitive to the actions of PGE_2. Additional experiments are needed to address these questions.

SUMMARY

Deficient TNF production is not related to deficiencies in, or defects or in signal transduction pathways which lead to TNF production, as other macrophage stimulants failed to correct the deficiency in TNF production. However, over-production of PGE_2 may be partly responsible for deficient TNF production and treatment with CFA corrects this imbalance. These results suggest that additional experiments are necessary to address the role of PGE_2 and deficient TNF production and how it correlates with diabetes development. Treatment with prostaglandins inhibitors such as monoclonal or polyclonal antibodies or indomethacin, starting at a time in which CFA is effective in preventing disease may help to further determine the roles of PGE_2 and TNF in BB rat diabetes development.

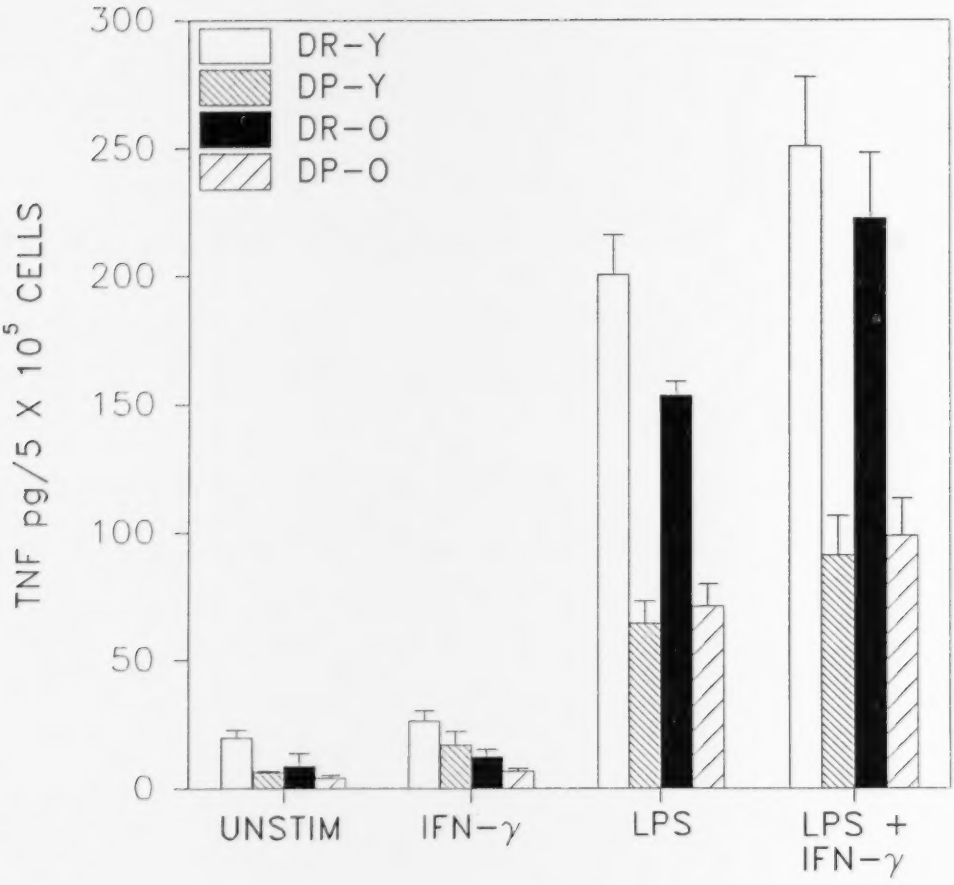

FIGURE 10.1
Tumour necrosis factor production after stimulation *in vitro*. Peritoneal cells from DR-YOUNG (diabetes-resistant) and DP-YOUNG (diabetes-prone) (ages 42-47 days), and DR-OLD and DP-OLD (ages 182-192 days) were incubated for 6 hours in control medium (UNSTIM), medium containing interferon γ (IFN-γ, 100 U/mL), lipopolysaccharide (LPS, 0.5 ug/mL), or IFN-γ + LPS. Values are Means ± S.E. for TNF levels in medium are shown for five DR-YOUNG and DP-YOUNG and three DR-OLD and DP-OLD rats in one experiment.

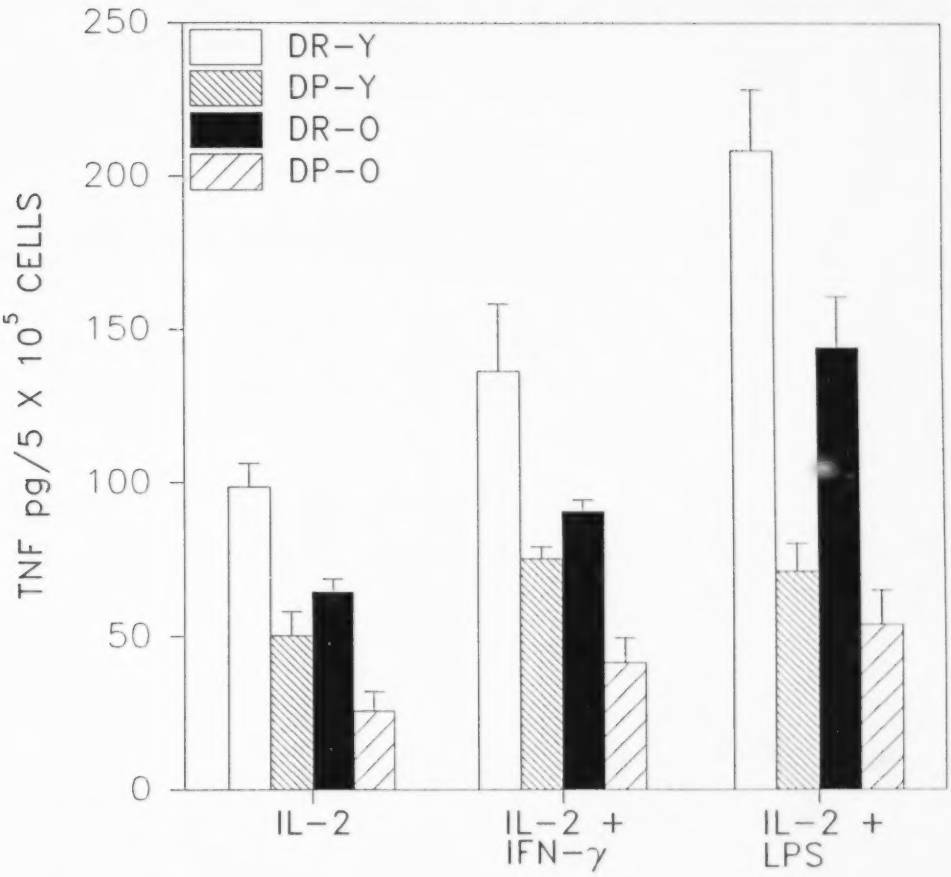

FIGURE 10.2
Tumour necrosis factor production after stimulation *in vitro* with helper T_1 lymphocyte cytokines. Peritoneal cells from DR-YOUNG (diabetes-resistant) and DP-YOUNG (diabetes-prone) (ages 42-47 days), and DR-OLD and DP-OLD (ages 182-192 days) were incubated for 6 hours in medium containing interleukin 2 (IL-2, 1000 U/mL), IL-2 + interferon γ (IFN-γ, 100 U/mL), or IL-2 + lipopolysaccharide (LPS, 0.5 ug/mL). Values are Means ± S.E. for TNF levels in medium are shown for five DR-YOUNG and DP-YOUNG and three DR-OLD and DP-OLD rats in one experiment.

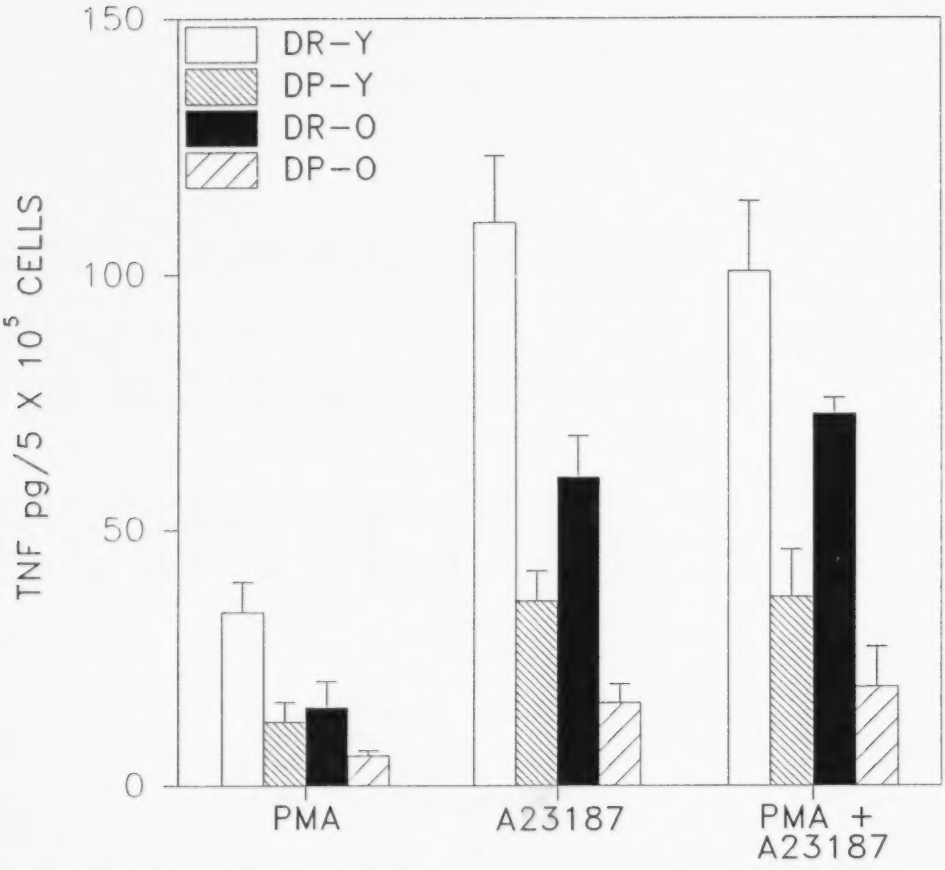

FIGURE 10.3
Tumour necrosis factor production after direct stimulation *in vitro* with protein kinase and calcium channel agonists. Peritoneal cells from DR-YOUNG (diabetes-resistant) and DP-YOUNG (diabetes-prone)(ages 42-47 days), and DR-OLD and DP-OLD (ages 182-192 days) were incubated for 6 hours in medium containing phorbol, 12-myristate, 13, acetate (PMA, 10 ng/mL), A23187 (5 uM), or PMA + A23187. Values are Means ± S.E. for TNF levels in medium are shown for five DR-YOUNG and DP-YOUNG and three DR-OLD and DP-OLD rats in one experiment.

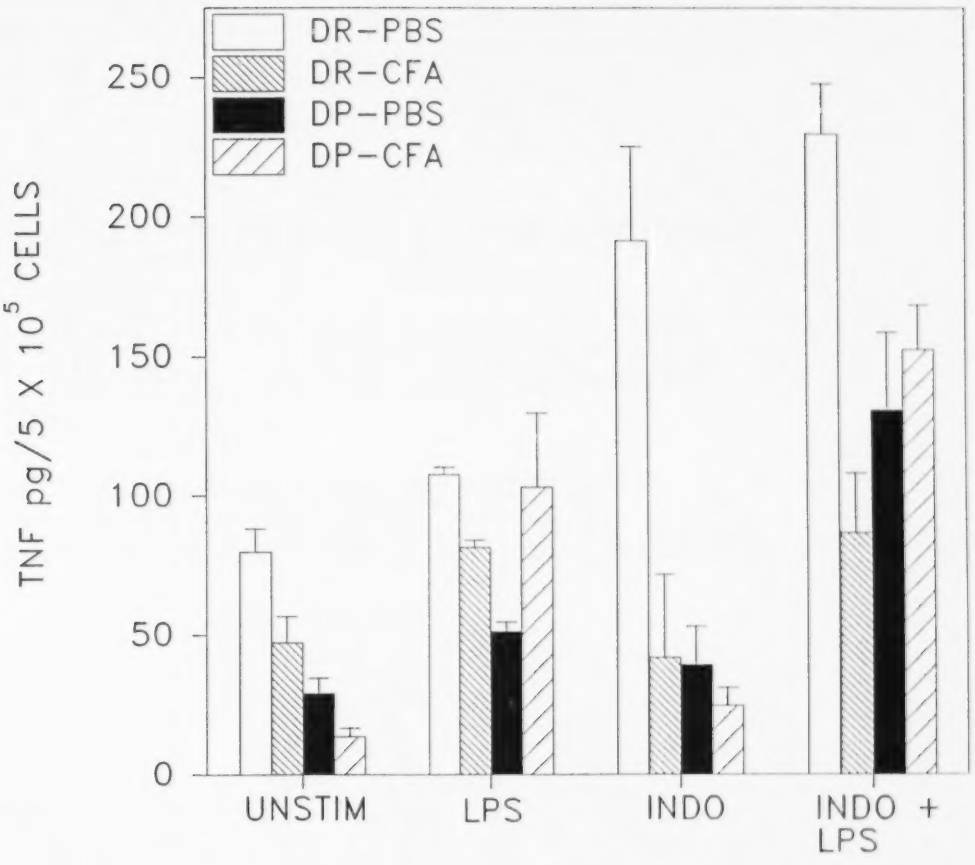

FIGURE 10.4
Tumour necrosis factor production after stimulation *in vitro* with lipopolysaccharide and a cyclooxygenase inhibitor, indomethacin. Peritoneal cells from DR (diabetes-resistant) and DP (diabetes-prone) (ages 40-42 days) were injected once with saline or complete Freund's adjuvant (CFA), then 10-14 days later peritoneal cells were collected and incubated for 6 hours in control medium (UNSTIM), medium containing lipopolysaccharide (LPS, 0.5 ug/mL), medium containing indomethacin (INDO, 5 ug/mL), or medium containing INDO + LPS. Values are Means ± S.E. for TNF levels in medium are shown for three DR and DP rats per group in one experiment.

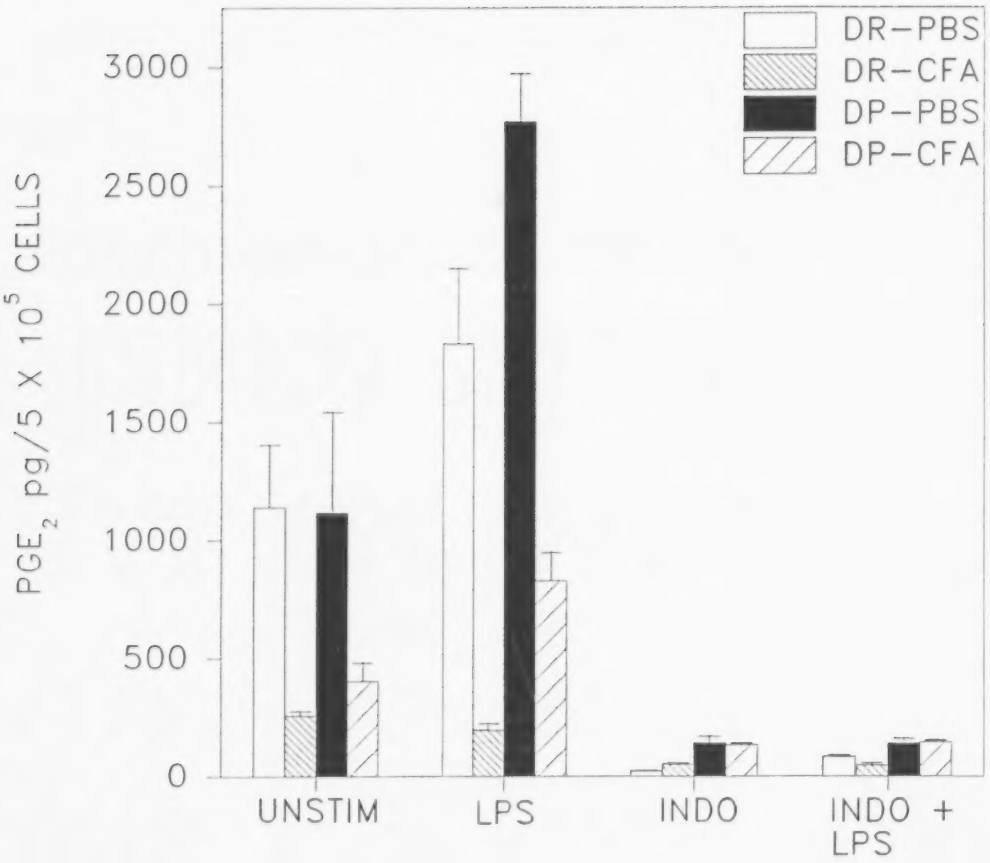

FIGURE 10.5
Prostaglandin E_2 production after stimulation *in vitro* with lipopolysaccharide and a cyclooxygenase inhibitor, indomethacin. Peritoneal cells from DR (diabetes-resistant) and DP (diabetes-prone)(ages 40-42 days) were injected once with saline or complete Freund's adjuvant (CFA), then 10-14 days later peritoneal cells were collected and incubated for 6 hours in control medium (UNSTIM), medium containing lipopolysaccharide (LPS, 0.5 ug/mL), medium containing indomethacin (INDO, 5 ug/mL), or medium containing INDO + LPS. Values are Means ± S.E. for prostaglandin E_2 (PGE_2) levels in medium (same medium used to assess TNF levels in **FIGURE 10.4**) are shown for three DR and DP rats per group in one experiment.

CHAPTER 11

MODULATION OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX CELL SURFACE ANTIGEN EXPRESSION ON BB RAT PERITONEAL CELLS AFTER TREATMENT WITH COMPLETE FREUND'S ADJUVANT

INTRODUCTION

Macrophage antigen presenting ability is mediated in the context of Class I and Class II major histocompatibility complex (MHC) antigens. Antigens presented by macrophage MHC result in distinct responses to that particular antigen by T lymphocytes (413). Tumour necrosis factor alpha (TNF-α) alone, or in concert with other cytokines such as interferon gamma (IFN-γ), can modulate MHC cell surface antigen expression on macrophages (324). As a result, the antigen-presenting ability of macrophages can be regulated.

Diabetes-prone (DP) BB rat lymphoid cells express increased amounts of Class II MHC RT1.B cell surface antigens compared to diabetes-resistant (DR) rats (72, 93, 95). This increase in Class II MHC expression suggests that lymphocytes are activated prior to disease development. Furthermore, macrophages found in the lesion at the onset of, and during insulitis express Class I and Class II MHC (RT1.B and RT1.D) antigens on their surfaces (93, 96). Together, increased expression of MHC antigens on lymphoid cells and macrophages suggest that MHC antigens are important in the development of diabetes. However, it has also been suggested that increases in Class II MHC RT1.D antigen cell surface expression associated with decreases in Class II MHC RT1.B antigen cell surface expression may prevent autoimmune disease by suppressing T lymphocyte responses to autoantigen (415, 416).

Class II MHC antigen expression on lymphoid cells, like non-lymphoid cells can be modulated by cytokines such as the interferons, TNF, and PGE_2 (325). Complete Freund's adjuvant is thought to prevent the onset of diabetes by altering macrophage function (273-276). However, it is not known whether non-specific immunostimulants such as CFA can modulate MHC antigen expression on macrophages and whether this modulation is associated with protection against diabetes.

Increased expression of MHC antigens on lymphoid cells, and on macrophages within the insulitis lesion may be important in diabetes development. Treatment of DP rats with CFA may modulate this expression, thereby preventing diabetes. Furthermore, old DP rats that never became diabetic may have escaped disease by having altered their expression of these cell surface antigens. This hypothesis was tested by measuring, by two-colour flow cytometry, Class I and Class II MHC cell surface antigen expressions on peritoneal cells from untreated, CFA-treated, and old non-diabetic DP rats to determine whether modulation of MHC cell surface antigen expression is associated with prevention of diabetes development.

RESULTS

Modulation of Major Histocompatibility Complex Antigen Expression by Complete Freund's Adjuvant

Two colour staining was done to determine whether CFA modulated Class I and/or Class II MHC antigen expression on peritoneal cells. Complete Freund's adjuvant significantly

decreased total OX-6 (RT1.B) and OX-17 (RT1.D) expressing peritoneal cells compared to untreated DR rats ($P \leq 0.001$ for RT1.B and RT1.D; **TABLE 11.1**). Complete Freund's adjuvant significantly increased total OX-6 positive peritoneal cells from DP rats compared to untreated DP rats ($P \leq 0.05$). Complete Freund's adjuvant treatment significantly increased OX-18 (Class I MHC) peritoneal cell expression compared to untreated DR rats ($P \leq 0.01$). Complete Freund's adjuvant significantly increased OX-18 peritoneal cell expression compared to old non-diabetic DP rats ($P \leq 0.05$).

Two colour staining was done to determine whether CFA modulated Class I and/or Class II MHC antigen expression on peritoneal macrophages. Complete Freund's adjuvant treatment significantly decreased Class II MHC (RT1.B and RT1.D) expression on peritoneal macrophages from DR rats compared to untreated DR rats ($P \leq 0.001$ for both RT1.B and RT1.D), without altering expression on peritoneal macrophages from CFA-treated DP rats (**TABLE 11.2**). Complete Freund's adjuvant treatment significantly decreased Class I MHC expressing peritoneal macrophages from DR rats compared to untreated DR rats ($P \leq 0.001$). Complete Freund's adjuvant treatment significantly increased Class II RT1.B and RT1.D expression on DP rat peritoneal macrophages compared to old non diabetic DP rats ($P \leq 0.005$ for RT1.B and $P \leq 0.001$ for RT1.D). Complete Freund's adjuvant significantly increased Class I MHC expressing peritoneal macrophages from DP rats compared to old non-diabetic DP rats ($P \leq 0.001$). Representative two-colour dot plots of OX-18$^+$/OX-43$^+$, OX-6$^+$/OX-43$^+$ cells and OX-17$^+$/OX-43$^+$ cells are presented in **Figures 11.1**, **11.2** and **11.3**, respectively.

OX-6 and OX-17 fluorescence intensity (FL2) (**TABLE 11.3**) from single histogram analysis and two-colour dot plot analysis (Ymean +/+ and Ymean +/—) was determined. No significant differences in FL2 and Ymean (+/—) of OX-6 were observed for DR and DP peritoneal cells. Ymean (+/+) was significantly lower for DR-CFA peritoneal cells compared to DR-OLD ($P \leq 0.05$), and Ymean (+/+) was significantly lower for DP-CFA compared to DP ($P \leq 0.05$). Fluorescence intensity was analyzed for OX-17. There was a significant decrease in OX-17 fluorescence intensity FL2 and Ymean (+/+) for peritoneal cells from DR-CFA rats compared to DR rats ($P \leq 0.01$), while there was a significant increase in Ymean (+/—) fluorescence intensity for DR-CFA compared to DR ($P \leq 0.05$) and DR-OLD (P 0.001). There was a significant decreases in Ymean (+/+) for DP-CFA compared to DP ($P \leq 0.05$). OX-18 fluorescence intensity (FL2) (**TABLE 11.4**) from single histogram analysis and two-colour dot plot analysis (Ymean +/+ and Ymean +/—) was determined. There were no significant differences in OX-18 fluorescence intensity (FL2) from single histogram analysis and two-colour dot plot analysis (Ymean +/+ and Ymean +/—).

Peak channel fluorescence decreased significantly for both OX-6 and OX-17 on peritoneal cells from both DR-CFA and DP-CFA rats (**TABLE 11.5**; $P \leq 0.05$ for OX-6 and OX-17 compared to DR and DP) and for DR-CFA compared to DR-OLD ($P \leq 0.005$ for OX-6, and $P \leq 0.05$ for OX-17). There were no significant differences in peak channel fluorescence in OX-18 stained peritoneal cells from DR-CFA and DP-CFA rats compared to controls.

DISCUSSION

Tumour necrosis factor and PGE_2 can modulate MHC cell surface antigen expression on lymphoid cells (325). Furthermore, modulation of MHC antigen expression and in

particular, aberrant expression of Class II MHC antigens is associated with autoimmune disease. Expression of I-E on macrophages is associated with suppression and whereas a lack of I-E expressing macrophages is associated with the potential for autoimmune disease (414, 415). There were no significant differences in RT1.B or RT1.D expressing DP rat peritoneal cells after CFA when compared to untreated animals, whereas cell numbers did increase with regards to old DP rats. However, fluorescence intensity of the double positive [Ymean (+/+)] cell population and peak channel fluorescence decreased significantly indicating that lower numbers of molecules were expressed on these cells. These results suggest that modulation of Class II MHC cell surface antigen expression may also contribute in the prevention of diabetes. However, at this time it is difficult to conclude whether this modulation of MHC antigen expression is associated directly with CFA or is a by-product of the actions of CFA, that is, mediated by increased TNF production and/or decreased PGE_2.

There were no significant changes in OX-18 expression on peritoneal macrophages from CFA-treated DP rats. This suggests that expression of this molecule is not associated with diabetes in the BB rats. However, it has been reported recently that lymphoid cells from human insulin-dependent diabetics and NOD mice express decreased amounts of Class I MHC molecules, thereby associating Class I MHC antigens with autoimmunity (415). These report also suggests that this decrease in Class I MHC antigen expression as associated with the inability to present antigen resulting in impaired development of cell tolerance. These findings are in contrast to those presented above.

SUMMARY

Complete Freund's adjuvant treatment of BB rats prevents the development of diabetes. As demonstrated in Chapter 3, one effect of CFA is to increase TNF production in response to stimulation. Tumour necrosis factor can modulate the expression of MHC antigens on macrophages. In this chapter, no differences in the number of cells expressing Class II MHC antigens could be seen, however, the intensity of Class II MHC antigen expression was decreased on peritoneal cells from CFA-treat DP rats. No differences in the numbers of cells and the number of Class I MHC molecules could be seen on peritoneal cells from CFA-treated DP rats. Overall, these results suggest that CFA directly or indirectly could alter Class II but not Class I MHC antigen expression on peritoneal cells, and this modulation of Class II MHC antigen expression may participate in prevention against diabetes.

TABLE 11.1

TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PERITONEAL CELLS FROM BB RATS

BB rat group	Percent Positive Cells		
	TTL OX-18$^+$	TTL OX-6$^+$	TTL OX-17$^+$
DR	89.6 ± 1.1	80.9 ± 1.0	80.1 ± 0.9
DP	85.4 ± 2.5	81.6 ± 1.4	81.8 ± 1.7
DR-OLD	80.4 ± 8.5	77.3 ± 4.1	75.7 ± 4.1
DP-OLD	85.0 ± 2.8	83.4 ± 1.9	83.9 ± 1.6
DR-CFA	96.7 ± 0.2**	68.2 ± 1.4***	67.7 ± 2.0***
DP-CFA	93.4 ± 0.8^{A}	87.0 ± 1.2*	86.5 ± 1.7

NOTE: Values are Means ± S.E. for percent positive cells from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compares DR-CFA to DR and DP-CFA to DP.

$^{A}P \leq 0.05$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 11.2

TWO-COLOUR FLOW CYTOMETRIC ANALYSIS OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION ON PERITONEAL CELLS FROM BB RATS

BB rat group	Percent Positive Cells					
	OX-18$^+$ OX-43$^-$	OX-18$^+$ OX-43$^+$	OX-6$^+$ OX-43$^-$	OX-6$^+$ OX-43$^+$	OX-17$^+$ OX-43$^-$	OX-17$^+$ OX-43$^+$
DR	15.6 ± 0.5	73.9 ± 1.1	7.5 ± 1.0	73.4 ± 0.6	7.8 ± 1.3	72.3 ± 0.7
DP	12.8 ± 1.2	72.5 ± 1.8	8.2 ± 1.6	73.4 ± 1.0	8.8 ± 1.9	73.0 ± 1.6
DR-OLD	15.3 ± 1.3	65.1 ± 7.2	6.6 ± 1.3	70.7 ± 3.5	6.1 ± 1.7	69.6 ± 3.6
DP-OLD	37.2 ± 3.2	47.8 ± 0.9	36.3 ± 6.6	47.1 ± 7.9	36.5 ± 3.0	47.5 ± 1.8
DR-CFA	34.2 ± 1.6^{*,C}	62.5 ± 1.6*	7.9 ± 0.5	60.3 ± 1.8*	6.4 ± 0.2	61.3 ± 2.2*
DP-CFA	15.4 ± 2.4^B	78.0 ± 2.6^D	9.8 ± 1.8^D	77.2 ± 2.7^A	9.2 ± 1.4^C	77.3 ± 2.6^D

NOTE: Values are Means ± S.E. for percent positive cells from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^*P \leq 0.001$ compares DR-CFA to DR and DP-CFA to DP.
$^AP \leq 0.05$, $^BP \leq 0.01$, $^CP \leq 0.005$, $^DP \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 11.3

FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION

BB rat group	Fluorescence Intensity					
	OX-6			OX-17		
	FL2	Ymean (+/+)	Ymean (+/—)	FL2	Ymean (+/+)	Ymean (+/—)
DR	267.3 ± 26.3	226.4 ± 16.9	23.8 ± 1.1	257.9 ± 27.5	216.6 ± 18.0	21.2 ± 0.9
DP	302.1 ± 33.5	245.0 ± 11.6	24.0 ± 1.3	294.6 ± 38.9	240.1 ± 15.7	23.0 ± 1.3
DR-OLD	263.5 ± 18.2	221.1 ± 11.0	23.2 ± 2.1	314.1 ± 20.2	225.1 ± 8.6	20.1 ± 0.5
DP-OLD	155.0 ± 40.3	185.6 ± 55.8	41.9 ± 15.7	170.9 ± 53.3	200.6 ± 64.2	41.6 ± 16.3
DR-CFA	208.0 ± 8.1	186.0 ± 4.6^{A}	25.2 ± 0.4	200.8 ± 5.0^{B}	177.6 ± 3.3^{B}	24.8 ± 0.1^{*,C}
DP-CFA	210.8 ± 10.1	176.8 ± 7.2*	23.5 ± 0.7	214.2 ± 15.7	176.6 ± 10.6*	20.0 ± 0.8

NOTE: Values are Means ± S.E. for fluorescence channels. Abbreviations used indicate mean channel fluorescence for single histogram analysis (FL2) and two-colour dot plot position (Ymean; where +/+ represents double positive cells and +/— represents single positive cells for OX-6 or OX-17 expression) analysis. Cells were obtained from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

$^{*}P \leq 0.05$ compares DR-CFA to DR and DP-CFA to DP. $^{A}P \leq 0.05$, $^{B}P \leq 0.01$, $^{C}P \leq 0.001$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 11.4

FLUORESCENCE INTENSITY OF BB RAT PERITONEAL CELLS STAINED FOR CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGEN EXPRESSION

BB rat group	Fluorescence Intensity FL2	OX-18 Ymean (+/+)	Ymean (+/—)
DR	283.7 ± 24.8	271.7 ± 22.1	26.3 ± 1.4
DP	339.2 ± 43.3	300.7 ± 22.8	30.2 ± 2.2
DR-O	303.9 ± 72.5	259.7 ± 25.0	22.0 ± 1.3
DP-O	151.2 ± 30.8	178.7 ± 34.5	44.4 ± 12.9
DR-CFA	237.1 ± 10.0	288.3 ± 12.3	31.7 ± 2.0[A]
DP-CFA	289.9 ± 15.5[A]	259.2 ± 10.6	30.8 ± 1.0

NOTE: Values are Means ± S.E. for fluorescence channels. Abbreviations used indicate mean channel fluorescence for single histogram analysis (FL2) and two-colour dot plot position (Ymean; where +/+ represents double positive cells and +/— represents single positive cells for OX-18) analysis. Cells were obtained from 7 young diabetes-resistant (DR) and diabetes-prone (DP), 4 old non-diabetic DR (DR-OLD) and DP (DP-OLD), and 3 complete Freund's adjuvant-treated DR (DR-CFA) and DP (DP-CFA) rats from one experiment.

[A]$P \leq 0.05$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

TABLE 11.5

PEAK CHANNEL FLUORESCENCE OF CLASS I AND CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS ON BB RAT PERITONEAL CELLS

BB rat group	Peak Channel Fluorescence OX-6	OX-17	OX-18
DR	323.0 ± 22.3	300.6 ± 20.7	367.7 ± 22.6
DP	310.6 ± 19.8	294.9 ± 16.4	357.8 ± 24.9
DR-OLD	272.7 ± 3.6	298.2 ± 23.3	338.3 ± 4.6
DP-OLD	115.0 ± 74.7	122.9 ± 87.0	116.7 ± 69.1
DR-CFA	227.4 ± 3.0^{*,B}	226.7 ± 1.2^{*,A}	350.5 ± 11.4
DP-CFA	249.4 ± 12.8*	236.6 ± 7.1*	340.2 ± 10.6

NOTE: Values are Means ± S.E. for peak channel fluorescence. Values were obtained from single colour histograms.

$^{*}P \leq 0.05$ compares DR-CFA to DR and DP-CFA to DP.
$^{A}P \leq 0.05$, $^{B}P \leq 0.005$ compares DR-CFA to DR-OLD and DP-CFA to DP-OLD.

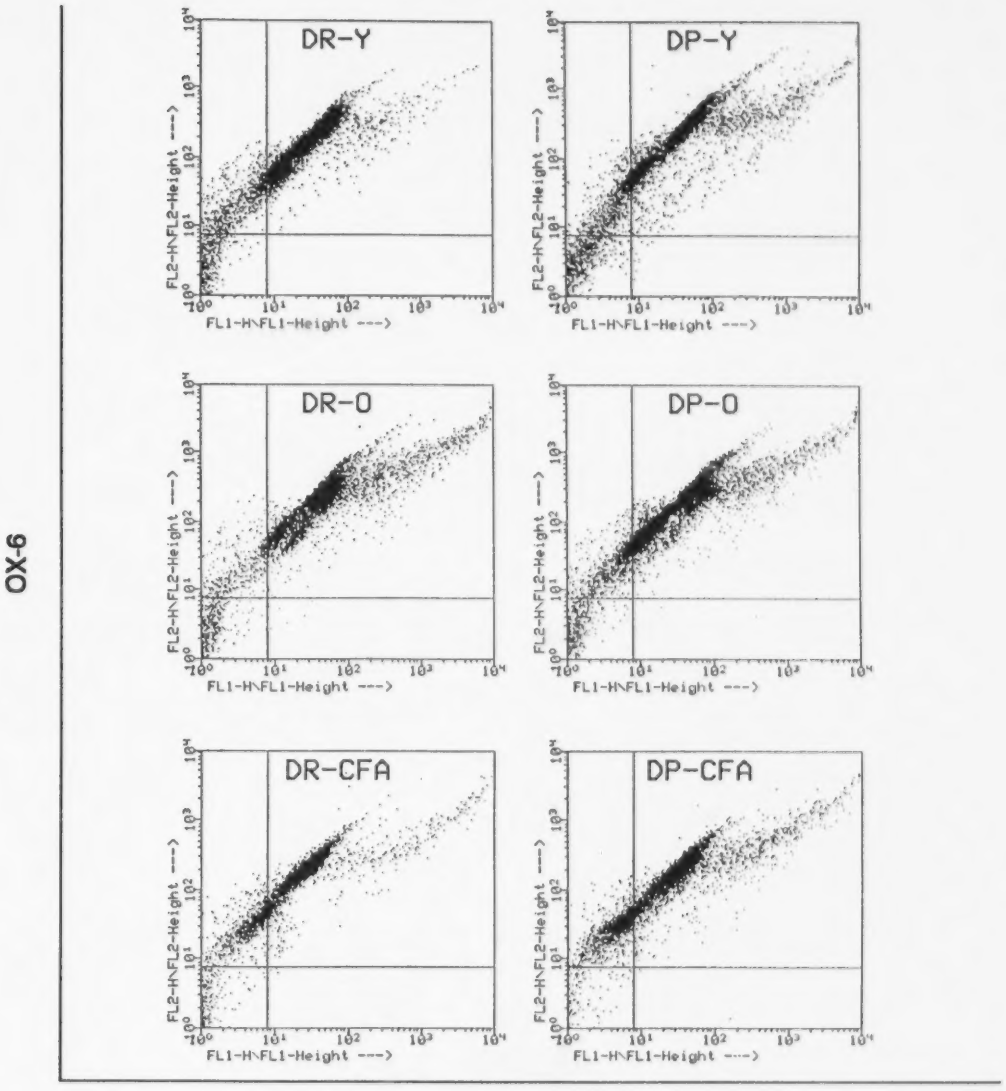

FIGURE 11.1
Representative two-colour dot-plots of flow cytometric analysis of antibody-labelled peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with OX-6 + goat anti-mouse-F(ab')$_2$-PE (FL2) and OX-43-FITC (FL1). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling. Controls are presented in Chapter 9.

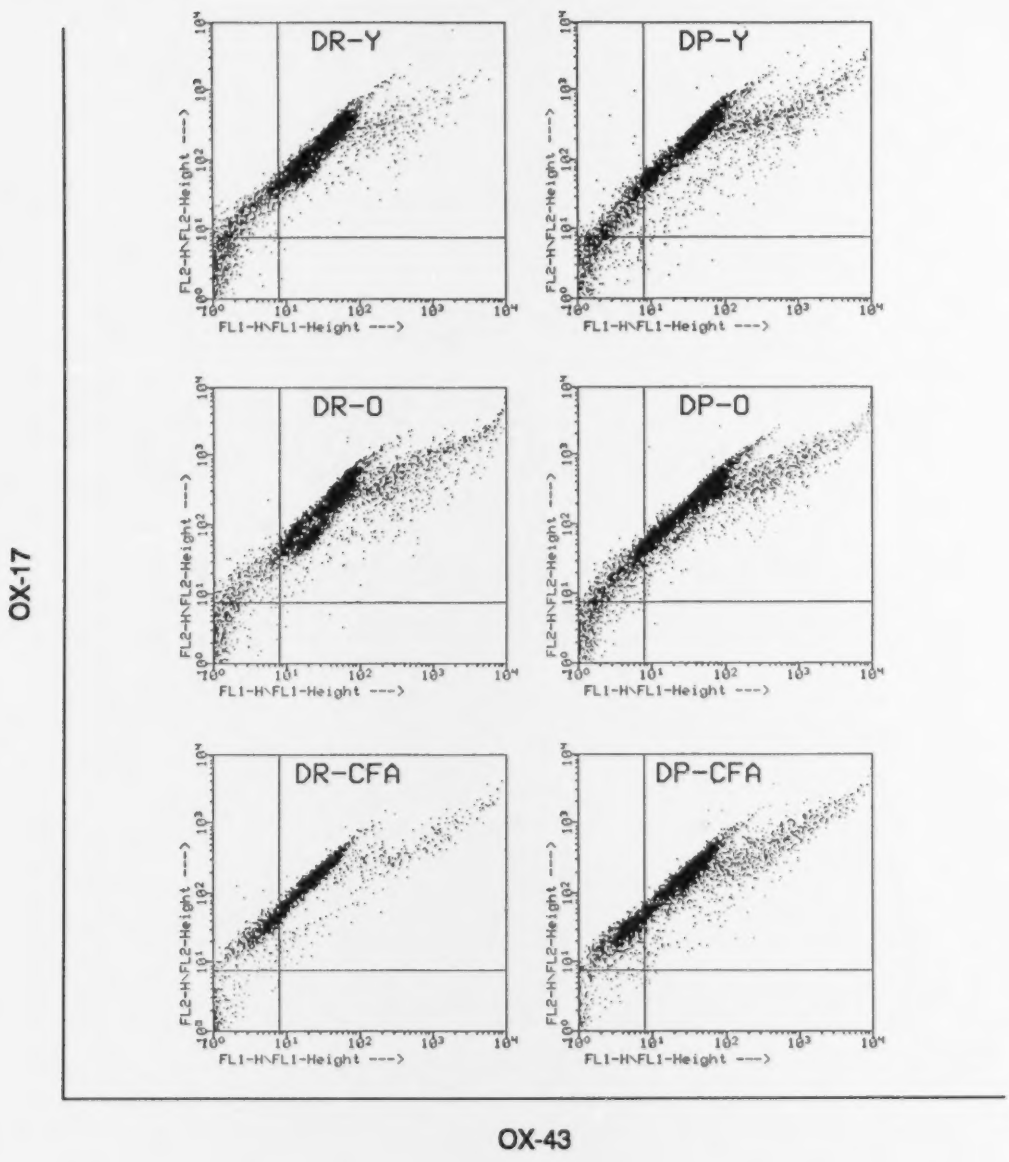

FIGURE 11.2
Representative two-colour dot-plots of flow cytometric analysis of antibody-labelled peritoneal cells from young (Y), old (O) and complete Freund's adjuvant (CFA)-treated DR diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with OX-17 + goat anti-mouse-F(ab')$_2$-PE (FL2) and OX-43-FITC (FL1). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling.

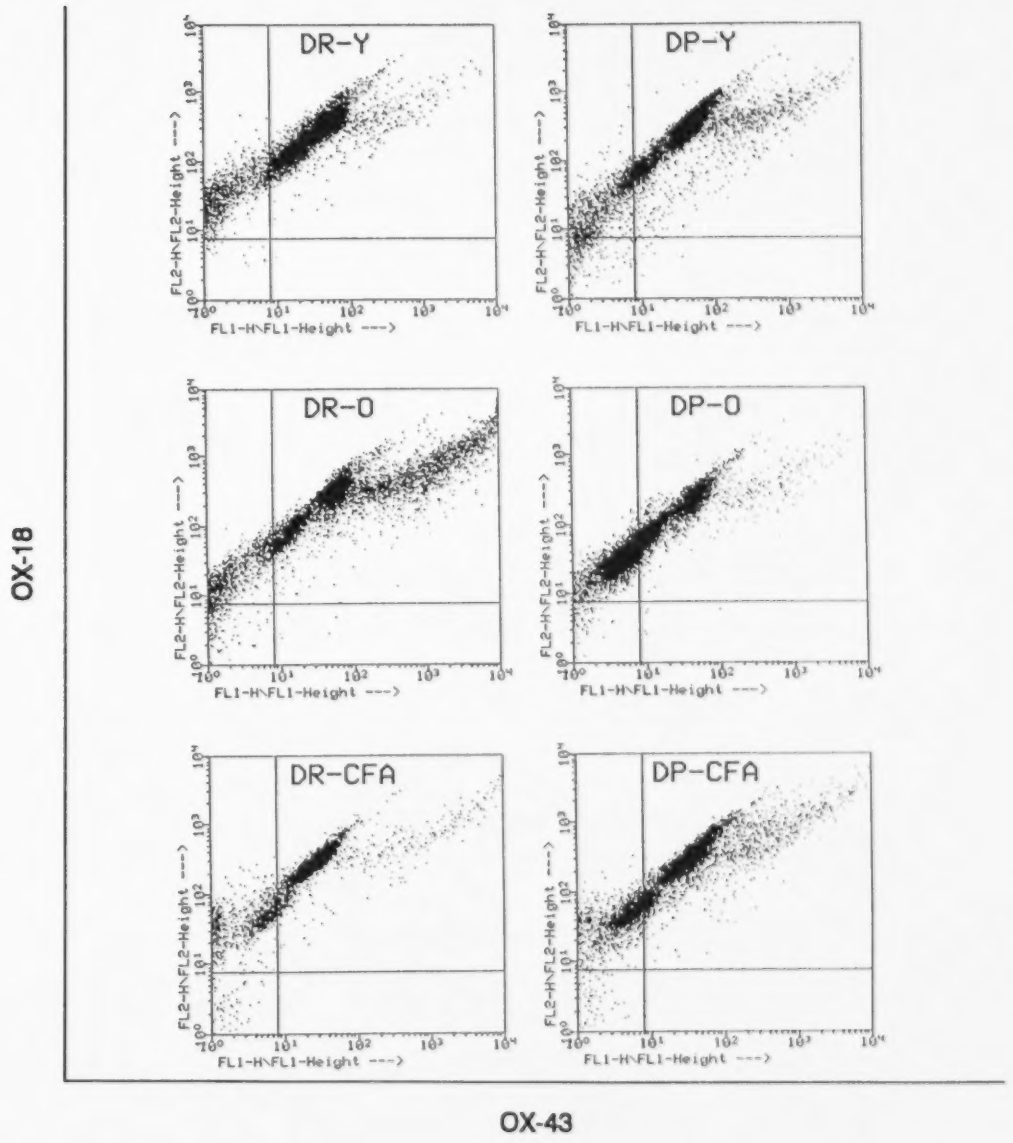

FIGURE 11.3
Representative two-colour dot-plots of flow cytometric analysis of antibody-labelled peritoneal cells from young (Y), old (O), and complete Freund's adjuvant (CFA)-treated diabetes-prone (DP) and diabetes-resistant (DR) rats. Cells were labelled with OX-18 + goat anti-mouse-F(ab')$_2$-PE (FL2) and OX-43-FITC (FL1). Vertical (x-axis; FL1) and horizontal (y-axis; FL2) markers were set to exclude unstained cells. Cells to the right of the vertical marker and above the horizontal marker are considered positive for labelling.

CHAPTER 12

DISCUSSION AND SUMMARY

DISCUSSION

Recent reports suggest that tumour necrosis factor (TNF) plays an important role in autoimmune diabetes development. Non-obese diabetic (NOD) mice or diabetes-prone (DP) BB rats treated chronically with TNF, or with agents which induce TNF, were protected against diabetes development (269, 270, 275, 300, 311, 328, 329, 333-335). These findings suggest that deficient TNF production may be an additional factor, together with specific Class II major histocompatibility complex (MHC) antigen haplotypes, lymphopenia, and a susceptibility to extensive pancreatic lymphocytic infiltration may act in concert to predispose BB rats to develop diabetes (3, 14, 16-21).

The goal of this thesis research was to examine the importance of TNF in diabetes susceptibility in BB rats. Experiments were done to determine whether TNF production was deficient, which cells produce TNF in a deficient/dysregulated manner, what factor(s) account for this deficiency/dysregulation, and by what mechanism(s) non-specific immunostimulation with complete Freund's adjuvant (CFA) protected against diabetes development.

The findings presented here show that TNF production by young DP rat peritoneal cells is deficient when compared to diabetes-resistant (DR) rats after stimulation *in vivo* or *in vitro*. Similarly, TNF production by young DP rat thymocytes appear deficient after stimulation *in vitro*. In contrast to deficient TNF production by peritoneal cells and thymocytes, young DP rat spleen cells over-produce TNF after stimulation *in vivo*. In another group of experiments, DP rats stimulated *in vivo* had serum TNF levels that were greater than those from DR, Wistar Furth and LEWIS rats. Furthermore, it was determined that the deficiency in DP rat peritoneal cell TNF production was not restricted to released TNF, but was also associated with cytosolic and membrane levels. Together, these results suggest that TNF production by DP rat lymphoid cells is dysregulated rather than deficient, and that threshold TNF levels by macrophages may be important for protecting against diabetes development.

Complete Freund's adjuvant protects DP rats and NOD mice against diabetes development (271-275). In a second series of experiments, it was shown that one way by which CFA appeared to protect against diabetes development in DP rats was to increase peritoneal cell and thymocyte TNF production, and decrease TNF production by spleen cells. This is in agreement with other experiments in which *in vitro* TNF production by peritoneal cells from DP rats that never developed diabetes were similar to those levels observed from DR rats. These results further support the notions that TNF is important in preventing diabetes, and that minimum threshold levels appear necessary to be preventative. To substantiate the finding that CFA protects against diabetes by increasing macrophage TNF production, anti-TNF antisera treatment of CFA-protected DP rats reversed the protection seen with CFA.

Diabetes-prone rat peritoneal cell TNF levels were deficient after culture *in vivo* or *in vitro* in response to interferon gamma (IFN-γ) or lipopolysaccharide (LPS), suggesting that

there may be deficiencies or defects in the expression of these receptors, or in signal transduction pathways associated with TNF gene induction. This was addressed by two series of experiments: measuring LPS receptor and CD14 expressions and measuring DP rat peritoneal cell TNF production after stimulation *in vitro* with T_{h1} cytokines, IFN-γ and interleukin 2 (IL-2), and protein kinase and Ca^{2+} channel agonists. Lipopolysaccharide receptor and CD14 expressions appear normal, however, TNF production by DP rat peritoneal cells remained deficient in response to these additional stimulatory agents suggesting that TNF production was suppressed rather than deficient/dysregulated.

In another series of experiments, the mechanism of suppression was investigated. Diabetes-prone BB rat peritoneal cells that were cultured *in vitro* with the cyclooxygenase inhibitor, indomethacin, or indomethacin + LPS, had increased levels of TNF which were similar to those levels observed from CFA-protected DP rat peritoneal cells. In addition, culture with indomethacin, or indomethacin + LPS significantly reduced prostaglandin E_2 (PGE_2) levels to those levels observed from CFA-protected DP rat peritoneal cells. This suggests that CFA may suppress PGE_2 production and/or reduce the sensitivity of DP rat peritoneal cells to the actions of PGE_2, thereby increasing TNF production. These results further suggest that a deficiency in TNF production by DP rat peritoneal cells can be attributed, in part to PGE_2 and that PGE_2 may be partly responsible for the susceptibility of DP rats to developing diabetes. A novel finding in this thesis research was the down-modulation of the peritoneal macrophage marker, OX-43, on peritoneal cells from CFA-treated DP and DR rats after culture with LPS. This finding suggests that CFA, may also act directly, or through other factors to induce increased differentiation/maturation of macrophages, thereby changing cytokine profiles and sensitivity to cytokines and other agents such as PGE_2.

Expression of Class II MHC cell surface antigens on DP rat leukocytes, and T and B lymphocytes appear to correlate with susceptibility to developing diabetes (45, 71-76). In addition Class II MHC cell surface antigen expression on macrophages also appear to correlate with DP rat diabetes and are found in the insulitis lesion (93, 96). Diabetes-prone rat peritoneal cell Class I and Class II MHC cell surface expressions was measured. No differences in cells numbers expressing Class I and Class II MHC cell surface antigens were found. However, there were decreases in the numbers of Class II, but not Class I MHC molecules on CFA-protected DP rat peritoneal cells. This suggests that an additional mechanism by which CFA protects against DP rat diabetes may be to down-regulate Class II MHC cell surface antigen expression directly, or through increased TNF production and/or decreased PGE_2 production or sensitivity. A decrease in Class II MHC cell surface antigen expression may disable macrophage antigen-presenting activity, and together with increased TNF production alone, or in combination with other factors, may induce a state of non-responsiveness in autoreactive lymphocytes.

Tumour necrosis factor was originally shown to be cytotoxic to islet cells *in vitro* (147, 151). However, administration of TNF, or agents which induce TNF production prevent diabetes in the NOD mouse and the BB rat (269, 270, 275, 300, 311, 328, 329, 333-335). This suggests that macrophage TNF production may be deficient or suppressed in these animals, and may be responsible for the progression of islet cell-specific autoreactivity by T lymphocytes. One explanation for TNF-mediated protection against diabetes is that by increasing TNF levels in diabetes-susceptible animals through the administration of

recombinant TNF or non-specific immunostimulatory agents may suppress autoreactive T lymphocytes. This is in accord with recent findings in TNF-α-treated mice in which mitogen responsiveness of T and B lymphocytes was inhibited, as well as T lymphocyte- and natural killer (NK) cell-mediated cytotoxicity, and the delayed type hypersensitivity (DTH) response *in vivo* (320). Therefore, administration of exogenous TNF, or agents which induce systemic TNF production may participate to prevent autoimmune disease is by inhibiting autoreactive T lymphocyte effector responses. However, it is still not clear by what mechanisms TNF inhibits T lymphocyte effector functions.

Many explanations can be presented for TNF- or CFA-mediated inhibition of diabetes. The three that will be discussed may be result of TNF inducing the production of additional macrophage factors, inducing macrophage differentiation/maturation, or TNF acting through its receptors to inhibit autoreactivity.

Tumour necrosis factor is considered an early response product of macrophages and is important in regulating itself and in initiating a cascade of other factors including interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 10 (IL-10), prostaglandins and granulocyte-macrophage colony stimulating factor (GM-CSF) (320, 367, 417, 418). These factors in turn, can inhibit TNF-α production (367, 417), while GM-CSF promotes TNF production (418).

In light of this, TNF can act as a macrophage differentiation/maturation factor (321). As demonstrated in this thesis research, peritoneal macrophages from CFA-treated DP rats have significantly decreased levels of the macrophage marker, OX-43. Therefore, CFA may act directly or through TNF and other factors to induce macrophage differentiation/ maturation. This explanation is supported by another finding in this thesis whereby CFA treatment significantly decreased prostaglandin E_2 (PGE_2) levels by DP rat peritoneal cells resulting in increased TNF levels. While DR rat PGE_2 were similar to those of DP rats, TNF levels were significantly greater. This suggests that DP rat peritoneal macrophages may be hyper-sensitive to PGE_2. The finding that GM-CSF derived macrophages are less sensitive to PGE_2-mediated suppression of TNF release than colony-stimulating factor 1 (CSF-1)-derived macrophages (419) suggests different states of macrophage differentiation. This conclusion is supported by the finding that the administration of GM-CSF to NOD mice prevents diabetes (270). However, BB rats treated with CSF-1 were not protected from developing diabetes (308) and may be due to the sensitivity of DP rat macrophages to prostaglandins (see below). Additional experiments are necessary to determine the maturation state of DP rat macrophages and whether there are differential effects of prostaglandins on TNF production.

Tumour necrosis factor may induce the production of PGE_2 in splenic macrophages, which in turn can act on autoreactive T lymphocytes to suppress their function. This is supported by a number of reports studying autoreactive T lymphocyte function in tumour-bearing mice whereby the addition of macrophages derived from tumour-bearing mice suppressed autoreactive T lymphocyte proliferation to syngeneic and to allo-antigens (425-427). The TNF-induced suppression of cellular immune responses described here and above (320), may be mediated by TNF-induced PGE_2 production than by TNF itself. These reports also indicate that macrophage Class II MHC antigen expression is decreased (420-422), similar to what was found in this thesis research.

Complete Freund's adjuvant has been shown to increase the number of Thy-1$^-$ Mac-1$^+$ natural suppressor cells in NOD spleens which can inhibit lymphocyte function (272, 275). Therefore, CFA may induce TNF which can inhibit autoreactivity. In addition, CFA can act directly or indirectly as a macrophage differentiation/maturation factor through the upregulation of GM-CSF production resulting in reduced DP rat macrophage PGE_2 sensitivity. Together, these actions could culminate in the increased production of TNF, even in the presence of PGE_2, which in turn elevates PGE_2 levels which then inhibit autoreactive T lymphocyte function. Prostaglandins preferentially inhibit T_{h1} lymphocytes without affecting T_{h2} lymphocyte function including IL-4 and IL-5, and possibly IL-10 production (423, 424). Prostaglandins affect T_{h1} lymphocyte cytokine production by increasing intracellular cyclic 3',5' adenosine monophosphate (cAMP) levels and inhibiting IL-2 and IFN-γ synthesis in a manner similar to PGE_2-mediated inhibition of macrophage TNF production (419). Because T_{h2} lymphocytes have higher constitutive cAMP levels, they are less affected by PGE_2 (423, 424). Additional support for inhibition of T_{h1} production by PGE_2 and possibly by IL-10 involves the role cAMP in inducing IL-1 which also favours T_{h2} proliferation (424-426).

Additional experiments will be necessary to determine the role of CFA in diabetes prevention. Most importantly, the characterization of phenotypic and functional changes in macrophages from CFA-treated animals may shed light on whether increased macrophage maturity and cytokine profiles are responsible for protection against diabetes, or whether the induction of splenic macrophage PGE_2 is necessary for inhibition of autoreactivity directly or through T_{h2} cytokines.

Lastly, TNF may act directly through its receptors to inhibit autoreactive T lymphocyte killing of islet cells. Tumour necrosis factor binds to two receptors termed TNFR55 and TNFR75 (427) to mediate its effects. TNFR55 is associated with cytotoxicity and cytostasis whereas TNFR75 is associated with proliferation and differentiation (427). These differences in function are thought to be mediated by accessing different, but overlapping signal transduction pathways (427). Another explanation for the possible inhibitory effects of TNF on autoreactive T lymphocytes is suggested by the ability of TNF to increase cAMP levels likely through G proteins (417, 427). This increase in cAMP is reminiscent of the actions of PGE_2 on T_{h1} lymphocytes described above. As a result, TNF may act directly through TNFR55 to suppress autoreactive T lymphocytes by inhibiting IL-2 and IFN-γ production. Furthermore, TNF can act through TNFR55 on macrophages to induce PGE_2 production (428), and together TNF and PGE_2 may suppress autoreactive T lymphocyte activity. Additional experiments will be necessary to understand the roles of TNFR in regulating autoreactive lymphocyte responses. Antibodies specific for either TNFR55 or TNFR75, and TNF mutants which preferentially bind to one or the other receptor preferentially, are available (429-431) and will assist in determining the function(s) of TNFR in immune regulation which could be extended to understand autoimmunity.

SUMMARY

The research presented in this thesis documents the potential protective effects of TNF against autoimmune disease. Increases in TNF production after CFA treatment may be important alone, or in conjunction with other effects induced by TNF. Increases in peritoneal cell TNF and the reduction of TNF in spleen cells after CFA treatment may be important in the prevention of diabetes. However, other factors may also participate

including the differentiation/maturation of macrophages resulting in reduced PGE_2 sensitivity early after activation may also be important in the induction of sufficient amounts of TNF. Furthermore, decreases in macrophage Class II MHC antigen expression may also participate in suppressing autoreactive T lymphocytes. Overall, the thesis research presented here provides the foundation to determine the role(s) of TNF, CFA, and other factors in preventing or inducing autoimmune disease. This research also suggests that there exists fundamental defects in DP rat macrophages that require further characterization.

CHAPTER 13

BIBLIOGRAPHY

1. **Nakhooda, A.F., Like, A.A., Chappel, C.I., Murray, F.T., and Marliss, E.B.** The Spontaneously Diabetic Wistar Rat: Metabolic and Morphological Studies. *Diabetes 26(2): 100-112, 1976.*

2. **Chappel, C.I., and Chappel, W.R.** The Discovery and Development of the BB Rat Colony: An Animal Model of Spontaneous Diabetes Mellitus. *Metabolism 32(7 Suppl. 1): 8-10, 1983.*

3. **Colle, E., Gutttmann, R.D., and Seemayer, T.** Spontaneous Diabetes Mellitus Syndrome in the Rat. I. Association with the Major Histocompatibility Complex. *J. Exp. Med. 154: 1237-1242, 1981.*

4. **Guttmann, R.D., Colle, E., Seemayer, T., and Michel, F.** Spontaneous Diabetes in the Rat-A Powerful Probe of the MHC and Non-MHC Immunogenetic Interactions. *Transplant. Proceed. XIV(3): 547-549, 1982.*

5. **Seemayer, T., Oligny, L.L., Tannenbaum, G.S., Goldman, H., and Colle, E.** Animal Model: Spontaneous Diabetes Mellitus in the BB Wistar Rat. *Am. J. Pathol. 101: 485-488, 1980.*

6. **Seemayer, T., Colle, E., Tannenbaum, G.S., Oligny, L.L., Guttmann, R.D. and Goldman, H.** Spontaneous Diabetes Mellitus Syndrome in the Rat. III. Pancreatic Alterations in Aglycosuric and Untreated Diabetic BB Wistar-Derived Rats. *Metabolism 32(7 Suppl. 1): 26-32, 1983.*

7. **Naji, A., Silvers, W.K., and Barker, C.F.** Autoimmunity and Type I (Insulin-Dependent) Diabetes-Mellitus. *Transplantation 36: 355-361, 1983.*

8. **Shunto, S.** Histological Study of Pancreas of BB/W Rats Before and After the Onset of Diabetes Mellitus: Significance of Eosinophilic Infiltration in Pancreatic Islets. *J. Saitama Med. Sch. 16: 365-363, 1989.*

9. **Dean, B.M., Walker, R., Bone, A.J., Baird, J.D., and Cooke, A.** Pre-Diabetes in the Spontaneously Diabetic BB/E Rat: Lymphocyte Subpopulations in the Pancreatic Infiltrate and Expression of Rat MHC Class II Molecules in Endocrine Cells. *Diabetologia 28: 464-466, 1985.*

10. **Tannenbaum, G.S., Colle, E., Wanamaker, L., Gurd, W., Goldman, H., and Seemayer, T.A.** Dynamic Time-Course Studies of the Spontaneously Diabetic BB Wistar Rat. II. Insulin-, Glucagon-, and Somatostatin-Reactive Cells in the Pancreas. *Endocrinology 10(6): 1880-1887, 1981.*

11. **Seemayer, T.A., Tannenbaum, G.S., Goldman, H., and Colle, E.** Dynamic Time-Course Studies of the Spontaneously Diabetic BB Wistar Rat. III. Light-Microscopic and Ultrastructural Observations of Pancreatic Islets of Langerhans. *Am. J. Pathol. 106: 237-249, 1982.*

12. **Watters, J.W.F., Locker, J.D., Kunz, H.W., and Gill III, T.J.** Polymorphism and Mapping of the Class II Genes in the Rat: *RT1.B, RT1.D,* and *RT1.H,* a New *DP-Like* Region. *Immunogenetics 26: 220-229, 1987.*

13. **Gill III, T.J., Kunz, H.W., Misra, D.N., and Hassett, A.L.C.** The Major Histocompatibility Complex of the Rat. *Transplantation 43(6): 773-785, 1987.*

14. **Colle, E., Guttmann, R.D., Seemayer, T.A., and Michel, F.** Spontaneous Diabetes Mellitus Syndrome in the Rat. IV. Immunogenetic Interactions of MHC and Non-MHC Components of the Syndrome. *Metabolism 32(7 Suppl. 1): 54-61, 1983.*

15. **Jackson, R.A., Buse, J.B., Rifai, R., Pelletier, D., Milford, E.L., Carpenter, C.B., Eisenbarth, G.S., and Williams, R.M.** Two Genes Required for Diabetes in BB Rats: Evidence from Cyclical Intercrosses and Backcrosses. *J. Exp. Med. 159: 1629-1636, 1984.*

16. **Colle, E., Guttmann, R.D., and Fuks, A.** Insulin-Dependent Diabetes Mellitus Is Associated with Genes That Map to the Right of the Class I RT1.A Locus of the Major Histocompatibility Complex of the Rat. *Diabetes 35: 454-458, 1986.*

17. **Colle, E., Guttmann, R.D., and Fuks, A.** Spontaneous Diabetes in the Rat. In: *The Immunology of Diabetes.* Ed. Jaworski, M.A.,, et al. Elsevier Science Publishers B.V. 1986. pp 21-28.

18. **Colle, E., Guttmann, R.D., Fuks, A., Seemayer, T.A., and Prud'homme, G.J.** Genetics of the Spontaneous Diabetic Syndrome: Interaction of MHC and Non-MHC-Associated Factors. *Mol. Biol. Med 3: 13-23, 1986.*

19. **Markholst, H., Eastman, S., Wilson, D., Andreasen, B.E., and Lernmark, Å.** Diabetes Segregates as a Single Locus in Crosses between Inbred BB Rat Prone or Resistant to Diabetes. *J. Exp. Med. 174: 297-300, 1991.*

20. **Jacob, H.J., Pettersson, A., Wilson, D., Mao, Y., Lernmark, Å, and Lander, E.S.** Genetic Dissection of Autoimmune Type I Diabetes in the BB Rat. *Nature Genetics 2: 56-60, 1992.*

21. **Colle, E., Fuks, A., Poussier, P., and Guttmann, R.D.** Polygenic Nature of Spontaneous Diabetes in the Rat: Permissive MHC Haplotype and Presence of the Lymphopenic Trait of the BB Rat Are Not Sufficient to Produce Susceptibility. *Diabetes 41: 1616-1623, 1992.*

22. **Fuks, A., Colle, E., and Guttmann, R.D.** Genetic Mapping of the Tumor Necrosis Factor (TNF-α) Locus in the Major Histocompatibility Complex, RT1, of the Spontaneously Diabetic Rat. *Diabetes 40(Suppl. 1): 142A, 1991.*

23. **Buse, J.B., Ben-Nun, A., Klein, K.A., Eisenbarth, G.S., Seidman, J.G., and Jackson, R.A.** Specific Class II Histocompatibility Gene Polymorphism in the BB Rats. *Diabetes 33: 700-703, 1984.*

24. **Kastern W., Dyrberg, T., Schøller, J., and Kryspin-Sørensen, I.** Restriction Fragment Polymorphisms in the Major Histocompatibility Complex of Diabetic BB Rats. *Diabetes 33: 807-809, 1984.*

25. **Buse, J.B., Rifai-Haddad, R., Lees, S., Taniguchi, H., Chaplin, D., Milford, E.M., Seidman, J.G., Eisenbarth, G.S., and Jackson, R.A.** Major Histocompatibility Complex Restriction Fragment Polymorphisms Define Three Diabetogenic Haplotypes in BB and BBN Rats. *J. Exp. Med. 162: 444-458, 1985.*

26. **Goldner-Sauvé, A.J., Fuks, A., and Guttmann, R.D.** Electrophoretic Comparison of Class II Histocompatibility Antigens From the Spontaneously Diabetic BB Rat and the serologically Identical Wistar Furth Rat. *Transplant. Proceed. XVII(3): 1827-1828, 1985.*

27. **Holowachuk, E.W., and Greer, M.K.** Unaltered Class II Histocompatibility Antigens and Pathogenesis of IDDM in BB Rats. *Diabetes 38: 267-271, 1989.*

28. **Chao, N.J., Timmerman, L., McDevitt, H.O., and Jacob, C.O.** Molecular Characterization of MHC Class II Antigens (β_1 domain) in the BB Diabetes-Prone and -Resistant Rat. *Immunogenetics 29: 231-234, 1989.*

29. **Dyrberg, T., Nakhooda, A.F., Baekkeskov, S., Lernmark, Å., Poussier, P., and Marliss, E.B.** Islet Cell Surface Antibodies and Lymphocyte Antibodies in the Spontaneously Diabetic BB Wistar Rat. *Diabetes 31: 278-281, 1982.*

30. **Elder, M., Maclaren, N., Riley, W., and McConnell, T.** Gastric Parietal Cell and Other Autoantibodies in the BB Rat. *Diabetes 31: 313-318, 1982.*

31. **Like, A.A., Appel, M.C., and Rossini, A.A.** Autoantibodies in the BB/W Rat. *Diabetes 31: 816-820, 1982.*

32. **Dean, B.M., Bone, A.J., Varey, A.-M., Walker, R., Baird, J.D., and Cooke, A.** Insulin Autoantibodies, Islet Cell Surface Antibodies and the Development of Spontaneous Diabetes in the BB/Edinburgh Rat. *Clin. Exp. Immunol. 69: 308-313, 1987.*

33. **Dyrberg, T. Poussier, P., Nakhooda, A.F., Marliss, E.B., and Lernmark, Å.** Humoral Immunity in the Spontaneously Diabetic BB Rat. *Metabolism 32(7 Suppl. 1): 87-91, 1983.*

34. **Dyrberg, T. Poussier, P., Nakhooda, A.F., Marliss, E.B., and Lernmark, Å.** Islet Cell Surface and Lymphocyte Antibodies Often Precede the Spontaneous Diabetes in the BB Rat. *Diabetologia 26: 159-165, 1984.*

35. **Bertrand, S. Vigeant, C., and Yale, J.-F.** Predictive Value of Lymphocyte Antibodies for the Appearance of Diabetes in BB Rats. *Diabetes 43: 137-142, 1993.*

36. **Baekkeskov, S., Dyrberg, T., and Lernmark, Å.** Autoantibodies to a 64-Kilodalton Islet Cell Protein Precede the Onset of Spontaneous Diabetes in the BB Rat. *Science 224: 1348-1350, 1984.*

37. **Tanguay, K.E., Amano, K., Hart, D.A., and Yoon, J.-W.** A Cytotoxic Autoantibody From the BB Rat Which Binds an Islet Cell Surface Protein. *Diabetes Res. Clin. Pract. 8: 23-28, 1990.*

38. **Kim, J., Richter, W., Aanstoot, H.-T., Shi, Y., Fu, Q., Rajotte, R., Warnock, G., and Baekkeskov, S.** Differential Expression of GAD_{65} and GAD_{67} in Human, Rat, and Mouse Pancreatic Islets. *Diabetes 42: 1799-1808, 1993.*

39. **Jackson, R., Rassi, N., Crump, T., Haynes, B., and Eisenbarth, G.S.** The BB Diabetic Rat: Profound T-Cell Lymphocytopenia. *Diabetes 30: 887-889, 1981.*

40. **Poussier, P., Nakhooda, A.F., Falk, J.A., Lee, C., and Marliss, E.B.** Lymphopenia and Abnormal Lymphocyte Subsets in the "BB" Rat: Relationship to the Diabetic Syndrome. *Endocrinology 110(5): 1825-1827, 1982.*

41. **Naji, A., Kimura, H., Silvers, W., and Barker, C.F.** Numerical and Functional Abnormality of T Suppressor Cells in Diabetic Rats. *Surgery 94(2): 235-241, 1983.*

42. **Yale, J.-F., Grose, M., and Marliss, E.B.** Time Course of the Lymphopenia in BB Rats: Relation to the Onset of Diabetes. *Diabetes 34: 955-959, 1985.*

43. **Woda, B.A., Like, A.A., Padden, C., and McFadden, M.L.** Deficiency of Phenotypic Cytotoxic-Suppressor T Lymphocytes in the BB/W Rat. *J. Immunol. 136(3): 856-859, 1986.*

44. **Prud'homme, G.J., Lapchak, P.H., Parfrey, N.A., Colle, E., and Guttmann, R.D.** Autoimmunity-Prone BB Rats Lack Functional Cytotoxic T Cells. *Cell. Immunol. 114: 198-208, 1988.*

45. **Hosszufalusi, N., Chan, E., Granger, G., and Charles, M.A.** Quantitative Analyses Comparing All Major Spleen Cell Phenotypes in BB and Normal Rats: Autoimmune Imbalance and Double Negative T Cells Associated with Resistant, Prone and Diabetic Animals. *J. Autoimmun. 5: 305-318, 1992.*

46. **Like, A.A., Guberski,, D., and Butler, L.** Diabetic Biobreeding/Worcester (BB/Wor) Rats Need Not be Lymphopenic. *J. Immunol. 136(9): 3254-3258, 1986.*

47. **Greiner, D.L., Handler, E.S., Nakano, K., Mordes, J.P., and Rossini, A.A.** Absence of the RT-6 T Cell Subset in Diabetes-Prone BB/W Rats. *J. Immunol. 136(1): 148-151, 1986.*

48. **Greiner, D.L., Angelillo, M., Mordes, J.P., Handler, E.S., Mojcik, C.F., Makamura, N., and Rossini, A.A.** Regulatory T Cell Control of Autoimmune Destruction of Beta Cells in the BB rat. *Adv. Exp. Med. Biol. 246: 379-385, 1988.*

49. **Crisá, L., Greiner, D.L., Mordes, J.P., MacDonald, R.G., Handler, E.S., Czech, M.P., and Rossini, A.A.** Biochemical Studies of RT6 Alloantigens in BB/Wor and Normal Rats: Evidence for Intact Unexpressed RT6^a Structural Gene in Diabetes-Prone BB Rats. *Diabetes 39: 1279-1288, 1990.*

50. **Angelillo, M., Greiner, D.L., Mordes, J.P., Handler, E.S., Nakamura, N., McKeever, U., and Rossini, A.A.** Absence of RT6$^+$ T Cells in Diabetes-Prone Biobreeding Worcester Rats is Due to Genetic and Cell Developmental Defects. *J. Immunol. 141(12): 4146-4151, 1988.*

51. **Angelillo, M., Greiner, D.L., Crisá, L., Kitagawa, Y., Heyderman, J.I., Mordes, J.P., and Rossini, A.A.** RT6$^+$ T-Cell Developmental Defects in BB/Wor Rats. In: *Frontiers in Diabetes Research. Lessons from Animal Diabetes III.* Ed. Shafrir, E. Smith-Gordon and Co. Ltd. 1991. pp 114-119.

52. **Thiele, H.-G., Koch, F., Haag, F., and Wurst, W.** Evidence for Normal Thymic Export of Lymphocytes and an Intact RT6^a Gene in RT6 Deficient Diabetes Prone BB-Rats. *Thymus 14: 136-143, 1989.*

53. **Crisá, L., Sarkar, P., Waite, D.J., Haag, F., Koch-Nolte, F., Rajan, T.V., Mordes, J.P., Handler, E.S., Thiele, H.-G., Rossini, A.A., and Greiner, D.L.** An RT6^a Gene is Transcribed and Translated in Lymphopenic Diabetes-Prone BB Rats. *Diabetes 42: 688-695, 1993.*

54. **Fangmann, J., Schwinzer, R., Hedrich, H.-J., Klöting, I., and Wonigeit, K.** Diabetes-Prone BB Rats Express the RT6 Alloantigen on Intestinal Intraepithelial Lymphocytes. *Eur. J. Immunol. 21: 2011-2015, 1991.*

55. **Greiner, D.L., Mordes, J.P., Handler, E.S., Angelillo, M., Nakamura, N., and Rossini, A.A.** Depletion of RT6$^+$ T Lymphocytes Induces Diabetes in Resistant BioBreeding/ Worcester (BB/W) Rats. *J. Exp. Med. 166: 461-475, 1987.*

56. **Doukas, J., and Mordes, J.P.** T Lymphocytes Capable of Activating Endothelial Cells in Vitro Are Present in Rats with Autoimmune Diabetes. *J. Immunol. 150(3): 1036-1046, 1993.*

57. **Lang, F., and Kastern, W.** The Gene For the T Lymphocyte Alloantigen, RT6, is Not Linked to Either Diabetes or Lymphopenia and is Not Defective in the BB Rat. *Eur. J. Immunol. 19: 1785-1789, 1989.*

58. **Like, A.A.** Depletion of RT6.1$^+$ T Lymphocytes Alone Is Insufficient to Induce Diabetes in Diabetes-Resistant BB/Wor Rats. *Am. J. Pathol. 136(3): 565-574, 1990.*

59. **Powrie, F., and Mason, D.** Subsets of Rat CD4$^+$ T Cells Defined by Their Differential Expression of Variants of the CD45 Antigen: Developmental Relationships and *In Vitro* and *In Vivo* Functions. *Curr. Topic Microbiol. Immunol. 159: 79-96, 1990.*

60. **Sharpshott, S.M., Bell, E.B., and Sarawar, S.R.** CD45R CD4 T Cell Subset-Reconstituted Nude Rats: Subset-Dependent Survival of Recipients and Bi-Directional Isoform Switching. *Eur. J. Immunol. 21: 993-1000, 1991.*

61. **Groen, H., Van Der Berk, J.M.M.M., Nieuwenhuis, P., and Kampinga, J.** Peripheral T Cells in Diabetes Prone (DP) BB Rats Are CD45R-Negative. *Thymus 14: 145-150, 1989.*

62. **Woda, B.A., and Jiang, Z.** Immunotherapy of Diabetes in the RT6-Depleted Diabetes-Resistant BB/Wor Rat. In: *Frontiers in Diabetes Research. Lessons from Animal Diabetes III.* Ed. Shafrir, E. Smith-Gordon and Co. Ltd. 1990 pp 14-18.

63. **Rothe, H., Schuller, I., Richter, G., Jongeneel, C.V., Kiesel, U., Diamantstein, T., Blankenstein, T., and Kolb, H.** Abnormal TNF Production in Prediabetic BB Rats is Linked to Defective CD45R Expression. *Immunology 77: 1-6, 1992.*

64. **Rozing, J., Coolen, C., Tielen, F.J., Weegenaar, J., Schuurman, H.-J., Greiner, D.L., and Rossini, A.A.** Islet Cells Surface Antibodies in the Spontaneously Diabetic BB Wistar Rat. *Thymus 14: 125-135, 1989.*

65. **Plamondon, C., Kottis, V., Brideau, C., Métroz-Dayer, M.-D., and Poussier, P.** Abnormal Thymocyte Maturation in Spontaneously Diabetic BB Rats Involves the Deletion of CD4$^-$8$^+$ Cells. *J. Immunol. 144(3): 923-928, 1990.*

66. **Georgiou, H.M., Lagarde, A.-C., and Bellgrau, D.** T Cell Dysfunction in the Diabetes-Prone BB Rat: A Role for Thymic Migrants That Are Not T Cell Precursors. *J. Exp. Med. 167: 132-148, 1988.*

67. **Bhatia, E., Buse, J.B., and Jackson, R.A.** T-Cell Antigen Receptor Alpha Chain Polymorphisms in Insulin-Dependent Diabetes. *J. Autoimmun. 1: 389-397, 1988.*

68. **Gold, D.P., and Bellgrau, D.** Identification of a Limited T-Cell Receptor β Chain Variable Region Repertoire Associated with Diabetes in the BB Rat. *Proc. Nat'l. Acad. Sci. (USA) 88: 9888-9891, 1991.*

69. **Bellgrau, D., Redd, J.M., and Sellins, K.S.** Peculiar T-Cell Signalling Does Not Preclude Positive Selection in the Diabetes-Prone BB Rat. *Diabetes 43: 47-52, 1994.*

70. **Haag, F., Lernmark, Å., Simrell, C., and Thiele, H.-G.** Analysis of T-Cell Surface Marker Profiles During the Postnatal Ontogeny of Normal and Diabetes-Prone Rats. *Transplant. Proceed. 25(5): 2831-2832, 1993.*

71. **Jiang, Z., Handler, E.S., Rossini, A.A., and Woda, B.A.** Immunopathology of Diabetes in the RT6-Depleted Diabetes-Resistant BB/Wor Rat. *Am. J. Pathol. 137: 767-777, 1990.*

72. **Francfort, J.W., Barker, C.F., Kimura, H., Silvers, W.K., Frohman, M., and Naji, A.** Increased Incidence of Ia Antigen-Bearing T Lymphocytes in the Spontaneously Diabetic BB Rat. *J. Immunol. 134(3): 1577-1582, 1985.*

73. **Francfort, J.W., Naji, A., Silvers, W.K., Tomaszewski, J., Woehrle, M., and Barker, C.F.** Immunologic Studies of the Prediabetic Stage in the Spontaneous Autoimmune Diabetes Mellitus of the BB Rat. *Transplantation 40(6): 698-701, 1985.*

74. **Francfort, J.W., Naji, A., Markmann, D.P., Silvers, W.K., and Barker, C.F.** "Activated" T-Lymphocyte Levels in the Spontaneously Diabetic BB Rat Syndrome. *Surgery 98 (2): 251-258, 1985.*

75. **Francfort, J.W., Naji, A., Silvers, W.K., and Barker, C.F.** Elevated Levels of A Lymphocyte Subset Accurately Predict the Diabetes State in the BB Rat. *Pancreas 2(2): 141-145, 1987.*

76. **Holowachuk, E.W., Greer, M.K., and Martin, D.R.** Elevated mRNA Levels of Major Histocompatibility Complex Class II Genes in Lymphocytes of Autoimmune BB Rats. *Diabetes 37: 1637-1640, 1988.*

77. **Bellgrau, D., Naji, A., Silvers, W.K., Markmann, J.F., and Barker, C.F.** Spontaneous Diabetes in BB Rats: Evidence for a T Cell Dependent Immune Response Defect. *Diabetologia 23: 359-364, 1982.*

78. **Naji, A., Silvers, W.K., Kimura, H., Bellgrau, D., Markmann, J.F., and Barker, C.F.** Analytical and Functional Studies on the T Cells of Untreated and Immunologically Tolerant Diabetes-Prone BB Rats. *J. Immunol. 130(5): 2168-2172, 1983.*

79. **Elder, M.E., and Maclaren, N.K.** Identification of Profound Peripheral T Lymphocyte Immunodeficiencies in the Spontaneously Diabetic BB Rat. *J. Immunol. 130(4): 1723-1731, 1983.*

80. **Jackson, R., Kadison, P., Rassi, N., Jegosothy, B., and Eisenbarth, G.S.** Lymphocyte Abnormalities in the BB Rat. *Metabolism 32(7 Suppl. 1): 83-86, 1983.*

81. **Prud'homme, G.J., Fuks, A., Colle, E., Seemayer, T.A., and Guttmann, R.D.** Immune Dysfunction in Diabetes-Prone BB Rats: Interleukin 2 Production and Other Mitogen-Induced Responses Are Suppressed by Activated Macrophages. *J. Exp. Med. 159: 463-478, 1984.*

82. **Woda, B.A., and Padden, C.** Mitogen Responsiveness of Lymphocytes From the BB/W Rat. *Diabetes 35: 513-516, 1986.*

83. **Weringer, E.J., and Woodland, R.T.** Defective Interleukin-2 Autocrine Regulation of T-Lymphocytes in the BB/Wor Diabetes-Prone Rat. In: *Frontiers in Diabetes Research. Lessons from Animal Diabetes III.* Ed. Shafrir, E. Smith-Gordon and Co. Ltd. 1990. pp 99-105.

84. **Woda, B.A., and Padden, C.** Biobreeding/Worcester (BB/Wor) Rats are Deficient in the Generation of Functional Cytotoxic T Cells. *J. Immunol. 139(5): 1514-1517, 1987.*

85. **Bellgrau, D., and Biron, C.A.** Cytotoxic T-Cell Precursors with Low-Level CD8 in the Diabetes-Prone Biobreeding Rat: Implications for Generation of an Autoimmune T-Cell Repertoire. *Proc. Nat'l. Acad. Sci. (USA) 87: 313-317, 1990.*

86. **Woda, B.A., and Biron, C.A.** Natural Killer Cell Number and Function in the Spontaneously Diabetic BB/W Rat. *J. Immunol. 137(6): 1860-1866, 1986.*

87. **Prud'homme, G.J., Fuks, A., Colle, E., and Guttmann, R.D.** Isolation of T-Lymphocyte Lines with Specificity for Islet Cell Antigens from spontaneously Diabetic (Insulin-Dependent) Rats. *Diabetes 33: 801-803, 1984.*

88. **Prud'homme, G.J., Fuks, A., Guttmann, R.D., and Colle, E.** T Cell Hybrids with Specificity for Islet Cell Antigens. *J. Immunol. 136(5): 1535-1536, 1986.*

89. **Prud'homme, G.J., Lapchak, P.H., Colle, E., Fuks, A., and Guttmann, R.D.** Islet Cell Recognition By T Lymphocytes in Rat Insulin-Dependent Diabetes Mellitus. In: *The Immunology of Diabetes.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 121-126.

90. **Prud'homme, G.J., Colle, E., Fuks, A., and Guttmann, R.D.** Major Histocompatibility Complex Restriction of T-Lymphocyte Responses to Islet Cell Antigens in IDDM Rats. *Diabetes 36(2): 237-239, 1987.*

91. **Diaz, J.-L., Ways, J., and Hammonds, P.** T-Lymphocyte Lines Specific for Glutamic Acid Decarboxylase (GAD) the 64K β-Cell Antigen of IDDM. *Diabetes 41: 118-121, 1992.*

92. **Lee, K.U., Kim, M.K., Amano, K., Pak, C.Y., Jaworski, M.A., Mehta, J.G., and Yoon, J.-W.** Preferential Infiltration of Macrophages During Early Stages of Insulitis in Diabetes-Prone BB Rats. *Diabetes 37: 1053-1058, 1988.*

93. **Hanenberg, H., Kolb-Bachofen, V., Kantwerk-Funke, G., and Kolb, H.** Macrophage Infiltration Precedes and is a Prerequisite for Lymphocytic Insulitis in Pancreatic Islets of Pre-Diabetic BB Rats. *Diabetologia 32: 126-134, 1989.*

94. **Kolb, H., Burkart, V., Appels, B., Hannenberg, H., Kantwerk-Funke, G., Kiesel, U., Funda, J., Schraermeyer, U., and Kolb-Bachofen, V.** Essential Contribution of Macrophages to Islet Cell Destruction *In Vivo* and *In Vitro. J. Autoimmun. 3 (Suppl.): 117-120, 1990.*

95. **Walker, R., Bone, A.J., Cooke, A., and Baird, J.D.** Distinct Macrophage Populations in Pancreas of Prediabetic BB/E Rats: Possible Role for Macrophages in Pathogenesis of IDDM. *Diabetes 37: 1301-1304, 1988.*

96. **Kolb-Bachofen, V., Schraermeyer, U., Hoppe, T., Hanenberg, H., and Kolb, H.** Diabetes Manifestation in BB Rats is Preceded by Pan-Pancreatic Presence of Activated Inflammatory Macrophages. *Pancreas 7(5): 578-584, 1992.*

97. **Voorbij, H.A.M., Jeucken, P.H.M., Kabel, P.J., De Haan, M., and Drexhage, H.A.** Dendritic Cells and Scavenger Macrophages in Pancreatic Islets of Prediabetic BB Rats. *Diabetes 38: 1623-1629, 1989.*

98. **Ziegler, A.-G., Erhard, J., Lampeter, E.F., Nagelkerken, L.M., and Standl, E.** Involvement of Dendritic Cells in Early Insulitis of BB Rats. *J. Autoimmun. 5: 571-579, 1992.*

99. **Tafuri, A., Bowers, W.E., Handler, E.S., Appel, M.C., Lew, R., Greiner, D., Mordes, J.P., and Rossini, A.A.** High Stimulatory Activity of Dendritic Cells from Diabetes-Prone BioBreeding/Worcester rats Exposed to Macrophage-Derived Factors. *J. Clin. Invest. 91: 2040-2048, 1993.*

100. **Damoiseaux, J.G.M.C., Huitinga, I., Dopp, E.A., and Dijkstra, C.D.** Expression of the ED3 Antigen on Rat Macrophages in Relation to Experimental Autoimmune Diseases. *Immunobiol. 184: 311-320, 1992.*

101. **Majino, G., Joris, I., Handler, E.S., Desemone, J., Mordes, J.P., and Rossini, A.A.** A Pancreatic Venular Defect in the BB/Wor Rat. *Am. J. Pathol. 128: 210-215, 1987.*

102. **Oschilewski, U., Kiesel, U., and Kolb, H.** Administration of Silica Prevents Diabetes in BB Rats. *Diabetes 34: 197-199, 1985.*

103. **Lee, K.U., Pak, C.Y., Amano, K., and Yoon, J.-W.** Prevention of Lymphocytic Thyroiditis and Insulitis in Diabetes-Prone BB Rats by Depletion of Macrophages. *Diabetologia 31: 400-402, 1988.*

104. **Kiesel, U., Oschilewski, M., Kantwerk, G., Maruta, M., Hananberg, H., Treichel, U., Kolb-Bachofen, V., Hartung, H.P., and Kolb, H.** Essential Role of Macrophages in the Development of Type I Diabetes in BB Rats. *Transplant. Proceed. XVIII(6): 1525-1527, 1986.*

105. **Amano, K., and Yoon, J.-W.** Studies on Autoimmunity for Initiation of β-Cell Destruction. V. Decrease of Macrophage-Dependent T Lymphocytes and Natural Killer Cytotoxicity in Silica-Treated BB Rats. *Diabetes 39: 590-596, 1990.*

106. **Nash, J.R., Everson, N.W., Wood, R.F.M., and Bell, P.R.** Effect of Silica and Carageenan on the Survival of Islet Allografts. *Transplantation 29(3): 206-208, 1980.*

107. **Martin, D.R., and Logothetopoulos, J.** Complement-Fixing Islet Cell Antibodies in the Spontaneously Diabetic BB Rat. *Diabetes 33: 93-96, 1984.*

108. **Laborie, C., Sal, P., Feutren, S.G., Debray-Sachs, M., Quiniou-Debrie, M.C., Poussier, P., Marliss, E.B., and Assan, R.** Time Course of Islet Cell Antibodies in Diabetic and Nondiabetic BB Rats. *Diabetes 34: 904-910, 1985.*

109. **Pipeleers, D., van de Winkel, M., Dyrberg, T., and Lernmark, Å.** Spontaneously Diabetic BB Rats Have Age-Dependent Islet β-Cell-Specific Surface Antibodies at Clinical Onset. *Diabetes 36: 1111-1115, 1987.*

110. **Knospe, S., Köhler, E., and Klöting, I.** Cell-Mediated Immune Reactions against Islets of Langerhans in Diabetes-Prone BB Rats. *Exp. Clin. Endocrinol. 89(3): 290-296, 1987.*

111. **Schröder, D., Wanka, H., Hehmke, B., Erika, B., Klöting, E., Knospe, S., and Schmidt, S.** Beta Cell Destruction of Pancreatic Islets Transplanted into Diabetic BB/OK Rats May Be Reflected by Increased Antibody-Mediated Anti-Islet Cytotoxicity. *Diabetes Res. 15: 27-32, 1990.*

112. **Hehmke, B., Lucke, S., Schröder, D., Klöting, I., and Kohnert, K.-D.** Complement-Dependent Antibody-Mediated Cytotoxicity in the Spontaneously Diabetic BB/OK Rat: Association with β Cell Volume Density. *Eur. J. Immunol. 20: 1091-1096, 1990.*

113. **Hehmke, B., Schröder, D., and Klöting, I.** Early Appearance of Complement-Dependent Antibody-Mediated Cytotoxicity (C'AMC) to Islet Cells in Serum of Diabetes-Prone BB/OK Rats. *Diabetes Res. 13: 183-186, 1990.*

114. **Hehmke, B., Schröder, D., Klöting, I., and Kohnert, K.-D.** Complement-Dependent Antibody Mediated Cytotoxicity (C'AMC) To Pancreatic Islet Cells in the Spontaneously Diabetic BB/OK Rat: Interference from Cell-Bound and Soluble Inhibitors. *J. Clin. Lab. Immunol. 35: 71-81, 1991.*

115. **Schröder, D., Schmidt, S., Klöting, I., and Hehmke, B.** Effect of Syngeneic Islet Antigen Administration on Complement-Dependent Antibody-Mediated Cytotoxicity of Islet Cells and Diabetes Onset in Diabetes-Prone BB/OK Rats. *Autoimmunity 14: 283-289, 1993.*

116. **Appels, B., Burkart, V., Kantwerk-Funke, G., Funda, J., Kolb-Bachofen, V., and Kolb, H.** Spontaneous Cytotoxicity of Macrophages Against Pancreatic Islet Cells. *J. Immunol. 142(11): 3803-3808, 1989.*

117. **Nagy, M.V., Chan, E.K., Teruya, M., Forrest, L.E., Likhite, V., and Charles, M.A.** Macrophage-Mediated Islet Cell Cytotoxicity in BB Rats. *Diabetes 38: 1329-1331, 1989.*

118. **Schwizer, R.W., Leiter, E.H., and Evans, R.** Macrophage-Mediated Cytotoxicity Against Cultured Pancreatic Islet Cells. *Transplantation 37(6): 539-544, 1984.*

119. **MacKay, P., Boulton, A., and Rabinovitch, A.** Lymphoid Cells of BB/W Diabetic Rats Are Cytotoxic to Islet Beta Cells *In Vitro*. *Diabetes 34: 706-709, 1985.*

120. **MacKay, P., Jacobson, J., and Rabinovitch, A.** Spontaneous Diabetes Mellitus in the Bio-Breeding/Worcester Rat: Evidence *In Vitro* for Natural Killer Cell Lysis of Islet Cells. *J. Clin. Invest. 77: 916-924, 1986.*

121. **Rabinovitch, A., MacKay, P., Baquerizo, H., and Pukel, C.** Identification of Lymphoid Cells Cytotoxic to Islet Cells in Diabetic BB/W Rats. In: *The Immunology of Diabetes.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 81-87.

122. **Pukel, C., Baquerizo, H., and Rabinovitch, A.** Interleukin 2 Activates BB/W Diabetic Rat Lymphoid Cells Cytotoxic to Islet Cells. *Diabetes 36: 1217-1222, 1987.*

123. **Koevary, S.B.** *In Vitro* Natural Killer Cell Activity in the Spontaneously Diabetic BB/Wor Rat: Effects of Serum on Lysis of Insulinoma Cells. *Diabetes Res. 8: 77-84, 1988.*

124. **Koevary, S.B.** *In Vitro* Lysis of Islet Cells by Lymphocytes From BB/Wor Rats IS Not Antibody-Mediated. *Diabetes Res. 13: 133-138, 1990.*

125. **Kitagawa, Y., Greiner, D.L., Reynolds, C.W., Ortaldo, J.R., Toccafondi, R., Handler, E.S., van der Meide, P.H., Mordes, J.P., and Rossini, A.A.** Islet Cells but Not Thyrocytes are Susceptible to Lysis by NK Cells. *J. Autoimmun. 4: 703-716, 1991.*

126. **Ellerman, K., Wrobleski, M., Rabinovitch, A., and Like, A.** Natural Killer Cell Depletion and Diabetes Mellitus in the BB/Wor Rat (Revisited). *Diabetologia 36: 596-601, 1993.*

127. **Woda, B.A., Handler, E.S., Greiner, D.L., Reynolds, C., Mordes, J.P., and Rossini, A.A.** T-Lymphocyte Requirement for Diabetes in RT6-Depleted Diabetes-Resistant BB Rats. *Diabetes 40: 423-428, 1991.*

128. **Wagner, L., Base, W., Wiesholzer, M., Sexi, V., Fürnainnm C., Lang, G., Froschauer, J., and Waldhäusl, W.** Incidence and Phenotype Restriction of Lymphoid BLT-Serine Protease Granules in Spontaneously Diabetes Prone BB Rats Compared with a Normal Rat Strain. *J. Autoimmun. 5: 581-590, 1992.*

129. **Jiang, Z., Makowiecki, J., Mordes, J.P., and Woda, B.A.** Cytolysin Gene Expression in the Islets of Diabetic Biobreeding/Worcester Rats. *J. Immunol. 152(1): 322-329, 1994.*

130. **Nakhooda, A.F., Sima, A.A.F., Poussier, P., and Marliss, E.B.** Passive Transfer of Insulitis From the "BB" Rat to the Nude Mouse. *Endocrinol. 109(4): 2264-2266, 1981.*

131. **Poussier, P., Nakhooda, A.F., Sima, A.A.F., and Marliss, E.B.** Passive Transfer of Insulitis and Lymphopenia in the BB Rat. *Metabolism 32(7 Suppl. 1): 73-79, 1983.*

132. **Rossini, A.A., Mordes, J.P., Williams, R.M., Pelletier, A.M., and Like, A.A.** Failure to Transfer Insulitis to Athymic Recipients Using BB/W Rat Lymphoid Tissue Transplants. *Metabolism 32(7 Suppl. 1): 80-82, 1983.*

133. **Koevary, S., Rossini, A., Stoller, W., and Chick, W.** Passive Transfer of Diabetes to the BB/W Rat. *Science 220: 727-728,* 1983.

134. **Koevary, S., Williams, D.E., Williams, R.M., and Chick, W.L.** Passive Transfer of Diabetes from BB/W to Wistar Furth Rats. *J. Clin. Invest. 75: 1904-1907, 1985.*

135. **Like, A.A., Weringer, E.J., Holdash, A., McGill, P., Atkinson, D., and Rossini, A.A.** Adoptive Transfer of Autoimmune Diabetes Mellitus in BioBreeding/Worcester (BB/W) Inbred and Hybrid Rats. *J. Immunol. 134(3): 1583-1587, 1985.*

136. **Handler, E.S., Mordes, J.P., Seals, J., Koevary, S., Like, A.A., Nakano, K., and Rossini, A.A.** Diabetes in the Bio-breeding/Worcester Rat: Induction and Acceleration by Spleen Cell-Conditioned Media. *J. Clin. Invest. 76: 1692-1694, 1985.*

137. **Logothetopoulos, J., Valiquette, N., MacGregor, D., and Hsia, T.** Adoptive Transfer of Insulitis and Diabetes in Neonates of Diabetes-Prone and Resistant Rats: Tissue Localization of Injected Blasts. *Diabetes 36: 1116-1123, 1987.*

138. **McKeever, U., Mordes, J.P., Greiner, D.L., Appel, M.C., Rosing, J., Handler, E.S., and Rossini, A.A.** Adoptive Transfer of Autoimmune Diabetes and Thyroiditis to Athymic Rats. *Proc. Nat'l. Acad. Sci. (USA) 87: 7618-7622, 1990.*

139. **Métroz-Dayer, M.-D., Mouland, A., Brideau, C., Duhamel, D., and Poussier, P.** Adoptive Transfer of Diabetes in BB Rats Induced by CD4 T Lymphocytes. *Diabetes 39: 928-932, 1990.*

140. **Edouard, P., Hiserodt, J.C., Plamondon, C., and Poussier, P.** $CD8^+$ T-Cells Are Required for Adoptive Transfer of the BB Rat Diabetic Syndrome. *Diabetes 42: 390-397, 1993.*

141. **Mandrup-Poulsen, T., Bendtzen, K., Nielsen, J.H., Bendixen, G., and Nerup, J.** Cytokines Cause Functional and Structural Damage to Isolated Islets of Langerhans. *Allergy 40: 424-429, 1985.*

142. **Bendtzen, K., Mandrup-Poulsen, T., Nerup, J., Dinarello, C.A., and Svenson, M.** Cytotoxicity of Human *p*I 7 Interleukin-1 for Pancreatic Islets of Langerhans. *Science 232: 1545-1547, 1986.*

143. **Mandrup-Poulsen, T., Bendtzen, K., Nerup, J., Dinarello, C.A., Svenson, M., and Nielsen, J.H.** Affinity-Purified Human Interleukin 1 is Cytotoxic to Isolated Islets of Langerhans. *Diabetologia 29: 63-67, 1986.*

144. **Mandrup-Poulsen, T., Spinas, G.A., Prowse, S.J., Hansen, B.S., Jørgensen, D.W., Bendtzen, K., Nielsen, J.H., and Nerup, J.** Islet Cytotoxicity of Interleukin 1: Influence of Culture Condition and Islet Donor Characteristics. *Diabetes 36: 641-647, 1987.*

145. **Nielsen, J.H., Mandrup-Poulsen, T., Spinas, G.A., Hansen, B.S., Mølvig, J., Nerup, J., and Bendtzen, K.** Possible Role of Interleukin- (IL-1) in the Pathogenesis of Insulin-Dependent Diabetes Mellitus (IDDM). In: *The Immunology of Diabetes Mellitus.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986 pp 95-103.

146. **Spinas, G.A., Hansen, B.S., Linde, S., Kastern, W., Mølvig, J., Mandrup-Poulsen, T., Dinarello, C.A., Nielsen, J.H., and Nerup, J.** Interleukin 1 Dose-Dependently Affects the Biosynthesis of (pro)insulin in Isolated Rat Islets of Langerhans. *Diabetologia 30: 474-480, 1987.*

147. **Mandrup-Poulsen, T., Bendtzen, K., Dinarello, C.A., and Nerup, J.** Potentiating Effects of Tumor Necrosis Factor on Interleukin-1 Mediated Pancreatic Beta-Cell Toxicity. In: *Monokines and Other Non-Lymphocytic Cytokines. Progress in Leukocyte Biology Volume 8.* Ed. Powanda, M.C., et al. Alan R. Liss, Inc. 1988. pp 291-295.

148. **Rabinovitch, A., Pukel, C., Baquerizo, H., and Mackay, P.** Immunological Mechanisms of Islet β-Cell Destruction: Cytotoxic Cells and Cytokines. In: *Frontiers in Diabetes Research. Lessons from Animal Diabetes II.* Ed. Shafrir, E., and Renold, A.E. John Libbey and Co. Ltd. 1988. pp 52-57.

149. **Rabinovitch, A., Pukel, C., Baquerizo, H., Pukel, C., and Sumoski, W.** Effects of Cytokines on Rat Pancreatic Islet Cell Monolayer Cultures: Distinction Between Functional and Cytotoxic Effects on Islet β-Cells. *Region. Immunol. 2(2): 77-82, 1989.*

150. **Mandrup-Poulsen, T., Bendtzen, K., Dinarello, C.A., and Nerup, J.** Human Tumor Necrosis Factor Potentiates Human Interleukin 1-Mediated Rat Pancreatic β-Cell Cytotoxicity. *J. Immunol. 139(12): 4077-4082, 1987.*

151. **Pukel, C., Baquerizo, H., and Rabinovitch, A.** Destruction of Rat Islet Cell Monolayers by Cytokines: Synergistic Interactions of Interferon-γ, Tumor Necrosis Factor, Lymphotoxin, and Interleukin 1. *Diabetes 37(1): 133-136, 1988.*

152. **Baquerizo, H., and Rabinovitch, A.** Interferon-Gamma Sensitizes Rat Pancreatic Islet Cells to Lysis by Cytokines and Cytotoxic Cells. *J. Autoimmun. 3(Suppl.): 123-130, 1990.*

153. **Soldevila, G., Buscema, M., Doshi, M., James, R.F.L., Bottazzo, F., and Pujol-Borrell, R.** Cytotoxic Effect of IFN-γ plus TNF-α on Human Islet Cells. *J. Autoimmun. 4: 291-306, 1991.*

154. **Ling, Z., In't Veld, P.A., and Pipeleers, D.G.** Interaction of Interleukin-1 With Islet β-Cells: Distinction Between, Indirect, Aspecific Cytotoxicity and Direct, Specific Functional Suppression. *Diabetes 42: 56-65, 1993.*

155. **Bendtzen, K., Buschard, K., Diamant, M., Horn, T., Svenson, M., and The Thyroid Cell Group.** Possible Role of IL-1, TNF-α, and IL-6 in Insulin-Dependent Diabetes Mellitus and Autoimmune Thyroid Disease. *Lymphokine Res. 8(3): 335-340, 1989.*

156. **Campbell, I.L., Cutri, A., Wilson, A., and Harrison, L.C.** Evidence for IL-6 Production by and Effects on the Pancreatic β-Cell. *J. Immunol. 143(4): 1188-1191, 1989.*

157. **Southern, C., Schulster, D., and Green, I.C.** Inhibition of Insulin Secretion from Rat Islets of Langerhans by Interleukin-6. *Biochem. J. 272: 243-245, 1990.*

158. **Mehta, V., Hao, W., Brooks-Worrell, B.M., and Palmer, J.P.** The Functional State of the β Cell Modulates IL-1 and TNF-Induced Cytotoxicity. *Lymphokine Cytokine Res. 12(4): 255-259, 1993.*

159. **Jiang, Z., and Woda, B.A.** Cytokine Gene Expression in the Islets of the Diabetic Biobreeding/Worcester Rat. *J. Immunol. 146(9): 2990-2994, 1991.*

160. **Yamada, K., Takane, N., Otabe, S., Inada, C., Inoue, M., and Nonaka, K.** Pancreatic β-Cell-Selective Production of Tumor Necrosis Factor-Induced by Interleukin-1. *Diabetes 42: 1026-1031, 1993.*

161. **Takane, N., Yamada, K., Otabe, S., Inoue, M., and Nonaka, K.** Interleukin-1 Induction of tumor Necrosis Factor-α mRNA and Bioactive Tumor Necrosis Factor-α in a Pancreatic β-Cell Line by a Mechanism Requiring No *De Novo* Protein Synthesis. *Biochem. Biophys. Res. Commun. 194(1): 163-169, 1993.*

162. **Koevary, S.B.** Effects of Transforming Growth Factor β (TGF-β) on Lymphocyte- and Cytokine-Mediated Destruction of Islet Cells in the BB/Wor Rat. *Lymphokine Cytokine Res. 10(5): 337-342, 1991.*

163. **Koevary, S.B.** Effects of Incubation of Islet Cells With Transforming Growth Factor Beta (TGF-β) on Their Susceptibility to Diabetic Lymphocyte Mediated Destruction in the Rat, *In Vitro. Diabetes Res. 18: 123-127, 1991.*

164. **Yamanaka, Y., Friess, H., Buchler, M., Beger, H.G., Gold, L.I., and Korc, M.** Synthesis and Expression of Transforming Growth Factor β-1, β-2, and β-3 in the Endocrine and Exocrine Pancreas. *Diabetes 42: 746-756, 1993.*

165. **Wogensen, L., Huang, X., and Sarvetnick, N.** Leukocyte Extravasation into the Pancreatic Tissue in Transgenic Mice Expressing Interleukin 10 in the Islets of Langerhans. *J. Exp. Med. 178: 175-185, 1993.*

166. **Sandler, S., and Welsh, N.** Interleukin-10 Stimulates Rat Pancreatic Islets *In Vitro* but Fails to Protect Against Interleukin-1. *Biochem. Biophys. Res. Commun. 195(2): 859-865, 1993.*

167. **Lee, M.-S., Wogensen, L., Shizuru, J., Oldstone, M.B.A., and Sarvetnick, N.** Pancreatic Islet Production of Murine Interleukin-10 Does Not Inhibit Immune-Mediated Tissue Destruction. *J. Clin. Invest. 93: 1332-1338, 1994.*

168. **Hughes, J.H., Easom, R.A., Wolf, B.A., Turk, J., and McDaniel, M.L.** Interleukin 1-Induced Prostaglandin E_2 Accumulation by Isolated Pancreatic Islets. *Diabetes 38: 1251-1257, 1989.*

169. **Dayer-Mêtroz, M.-D., Wolheim, C.B., Seckinger, P., and Dayer, J.-M.** A Natural Interleukin 1 (IL-1) Inhibitor Counteracts the Inhibitory Effect of IL-1 on Insulin Production in Cultured Rat Pancreatic Islets. *J. Autoimmun. 2: 163-171, 1989.*

170. **Skoskiewicz, M.J., Colvin, R.B., Schneeberger, E.E., and Russell, P.S.** Widespread and Selective Induction of Major Histocompatibility Complex-Determined Antigens *In Vivo* by γ Interferon. *J. Exp. Med. 162: 1645-1664, 1985.*

171. **Johnson, D.R., and Pober, J.S.** Tumor Necrosis Factor Regulation of Major Histocompatibility Complex Gene Expression. *Immunol. Res. 10: 141-155, 1991.*

172. **Hanafusa, T., Fujino-Kurihara, H., Miyazaki, A., Yamada, K., Nakajima, H., Miyagama, J.-I., Kono, N., Nokada, K., and Tarui, S.** Expression of I-A Antigen on Pancreatic B Cells in NOD Mice. In: *The Immunology of Diabetes Mellitus.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 127-130.

173. **Bottazzo, G.F., Dean, B.M., McNally, J.M., MacKay, E.H., Swift, P.G.F., and Gamble, D.R.** In Situ Characterization of Autoimmune Phenomena and Expression of HLA Molecules in the Pancreas in Diabetic Insulitis. *New Engl. J. Med. 313(6):353-360, 1985.*

174. **Campbell, I.L., Wong, G.H.W., Schrader, J.W., and Harrison, L.C.** Interferon-γ Enhances the Expression of the Major Histocompatibility Class I Antigens on Mouse Pancreatic Beta Cells. *Diabetes 34(11): 1205-1209, 1985.*

175. **Wright Jr., J.R., Lacy, P.E., Unanue, E.R., Muszynski, C., and Haupfeld, V.** Interferon-Mediated Induction of Ia Antigen Expression on Isolated Murine Whole Islets and Dispersed Islet Cells. *Diabetes 35(10): 1174-1177, 1986.*

176. **Walker, R., Cooke, A., Bone, A.J., Dean, B.M., van der Meide, P., and Baird, J.D.** Induction of Class II MHC Antigens *In Vitro* on Pancreatic B Cells Isolated From BB/E Rats. *Diabetologia 29: 749-751, 1986.*

177. **Campbell, I.L., Bizilj, K., Colman, P.G., Tuch, B.E., and Harrison, L.C.** Interferon-γ Induces the Expression of HLA-A, B, C but Not HLA-DR on Human Pancreatic β-Cells. *J. Clin. Endocrinol. Metabol. 62(6): 1101-1109, 1986.*

178. **Foulis, A.K., and Farquharson, M.A.** Aberrant Expression of HLA-DR Antigens by Insulin-Containing β-Cells in Recent-Onset Type I Diabetes Mellitus. *Diabetes 35: 1215-1224, 1986.*

179. **Soldevila, G., Doshi, M., James, R., Lake, S.P., Sutton, R., Gray, D., Bottazzo, G.F., and Pujol-Borrell, R.** HLA DR, DP, and DQ Induction in Human Islet β Cells by the Cytokine Combination of IFN-γ + TNF-α. *Autoimmunity 6: 307-317, 1990.*

180. **Pujol-Borell, R., Todd, I., Adolf, G.R., Feldmann, M., and Bottazzo, G.F.** *In Vivo* and *In Vitro* Demonstration of HLA Class II Products on Human Islet Beta Cells. In: *The Immunology of Diabetes Mellitus.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 89-94.

181. **Pujol-Borell, R., Todd, I., Doshi, M., Bottazzo, G.F., Sutton, R., Gray, D., Adolf, G.R., and Feldmann, M.** HLA Class II Induction in Human Islet Cells by Interferon-γ Plus Tumour Necrosis Factor or Lymphotoxin. *Nature 326: 304-306, 1987.*

182. **Campbell, I.L., Harrison, L.C., Colman, P.G., Papaioannou, J., and Asshcroft, R.G.** Expression of Class I MHC Proteins on RINm5F Cells Is Increased by Interferon-γ and Lymphokine-Conditioned Medium. *Diabetes 35(11): 1225-1228, 1986.*

183. **van Vliet, E., Molenaar, J.L., Tuk, C.W., Bruining, G.J., and de Vries, R.R.P.** Recombinant γ Interferon Induces Class II Major Histocompatibility Complex Antigens on Insulinoma Cells. *Tissue Antigens 29: 195-200, 1987.*

184. **Markmann, J.F., Hickey, W.F., Kimura, H., Woehrle, M., Barker, C.F., and Naji, A.** Gamma Interferon Induces Novel Expression of Ia Antigens by Rat Pancreatic Islet Endocrine Cells. *Pancreas 2(3): 258-261, 1987.*

185. **Ono, S.J., Issa-Chergui, B., Colle, E., Seemayer, T., Guttmann, R.D., and Fuks, A.** Class I Major Histocompatibility Complex Gene Expression is Enhanced in the Pancreatic Islets of Prediabetic BB Rats. *Transplant. Proceed. XIX(3): 3187-3189,* 1987.

186. **Ono, S.J., Colle, E., Guttmann, R.D., Ullman, H., and Fuks, A.** In Vitro Studies on the Inducibility of MHC Gene Expression in the Rat Insulinoma Cell Line (RINm5F). *Transplant. Proceed. XIX(3): 2997-2999, 1987.*

187. **Ono, S.J., Issa-Chergui, B., Colle, E., Guttmann, R.D., Seemayer, T., and Fuks, A.** Enhanced MHC Class I Heavy-Chain Gene Expression in Pancreatic Islets. *Diabetes 37: 1411-1418, 1988.*

188. **Ono, S.J., B., Colle, E., Guttmann, R.D., and Fuks, A.** Interferon-γ Induces Transcription and Differential Expression of MHC Genes in Rat Insulinoma Cell Line RINm5F. *Diabetes 38: 911-916, 1989.*

189. **Campbell, I.L., Oxbrow, L., and Harrison, L.C.** Interferon-γ: Pleiotropic Effects on a Rat Pancreatic Beta Cell Line. *Mol. Cell. Endocrinol. 52: 161-167, 1987.*

190. **Foulis, A.K., Farquharson, M.A., and Hardman, R.** Aberrant Expression of Class II Major Histocompatibility Complex Molecules by B Cells and Hyperexpression of Class I Major Histocompatibility Complex Molecules by Insulin Containing Islets in Type 1 (Insulin-Dependent) Diabetes Mellitus. *Diabetologia 30: 333-334, 1987.*

191. **Issa-Chergui, B., Goldner-Sauvé, A., Prud'homme, G.J., Lapchak, P.H., van der Meide, P.H., and Seemayer, T.A.** Class I and II Major Histocompatibility Complex Gene Product Expression by a Rat Insulinoma Cell Line In Vitro Following Exposure to Gamma Interferon. *Diabetologia 31: 675-680, 1988.*

192. **Campbell, I.L., Oxbrow, L., West, J., and Harrison, L.C.** Regulation of MHC Protein Expression in Pancreatic β-Cells by Interferon-γ and Tumor Necrosis Factor-α. *Mol. Endocrinol. 2: 101-107, 1988.*

193. **Naji, A., Silvers, W.K., Bellgrau, D., Anderson, A.O., Plotkin, S., and Barker, C.F.** Prevention of Diabetes in Rats by Bone Marrow Transplantation. *Ann. Surgery 194: 328-338, 1981.*

194. **Naji, A., Silvers, W.K., Bellgrau, D., and Barker, C.F.** Spontaneous Diabetes in Rats: Destruction of Islets is Prevented by Immunological Tolerance. *Science 213: 1390-1392, 1981.*

195. **Naji, A., Silvers, W.K., Kimura, H., Bellgrau, D., Anderson, A.O., and Barker, C.F.** Correction of the Peripheral T-Cell Functional Microenvironmental Defects in BB Rats by Bone Marrow Transplantation. *Transplant. Proceed. XV(1): 1424-1426, 1983.*

196. **Naji, A., Silvers, W.K., Kimura, H., Anderson, A.O., and Barker, C.F.** Influence of Islet and Bone Marrow Transplantation on the Diabetes and Immunodeficiency of BB Rats. *Metabolism 32(7 Suppl. 1): 62-68, 1983.*

197. **Naji, A., Bellgrau, D., Anderson, A., Silvers, W.K., and Barker, C.F.** Transplantation of Islets and Bone Marrow Cells to Animals with Immune Insulitis. *Diabetes 31 (Suppl. 4): 84-89, 1982.*

198. **Scott, J., Engelhard, V.H., Curnow, R.T., and Benjamin, D.C.** Prevention of Diabetes in BB Rats. I. Evidence Suggesting a Requirement for Mature T Cells in Bone Marrow Inoculum of Neonatally Injected Rats. *Diabetes 35: 1034-1040, 1986.*

199. **Scott, J., Engelhard, V.H., and Benjamin, D.C.** Prevention of Diabetes in the BB Rat By Injection of Major Histocompatibility Complex- (MHC-) Compatible Bone Marrow Cells. *Adv. Exp. Med. Biol. 246: 387-395, 1988.*

200. **Nakano, K., Mordes, J.P., Handler, E.S., Greiner, D.L., and Rossini, A.A.** Role of Host Immune System in BB/Wor Rat: Predisposition to Diabetes Resides in the Bone Marrow. *Diabetes 37: 520-525, 1988.*

201. **Like, A.A., Kislauskis, E., Williams, R.M., and Rossini, A.** Neonatal Thymectomy Prevents Spontaneous Diabetes Mellitus in the BB/W Rat. *Science 216: 644-646, 1982.*

202. **Francfort, J.W., Naji, A., Silvers, W.K., and Barker, C.F.** The Influence of T-Lymphocyte Precursor Cells and Thymus Grafts on the Cellular Immunodeficiencies of the BB Rat. *Diabetes 34: 1134-1138, 1985.*

203. **Scott, J., Engelhard, V.H., and Benjamin, D.C.** Bone Marrow Irradiation Chimeras in the BB Rat: Evidence Suggesting Two Defects Leading to Diabetes and Lymphopenia. *Diabetologia 30: 774-781, 1987.*

204. **Georgiou, H.M., and Bellgrau, D.** Modulation of Insulitis Following Thymus Transplantation. *Transplant. Proceed. 21(1): 215-217, 1989.*

205. **Georgiou, H.M., and Bellgrau, D.** Thymus Transplantation and Disease Prevention in the Diabetes-Prone Bio-Breeding Rat. *J. Immunol. 142(10): 3400-3405, 1989.*

206. **Brayman, K.L., Deaton, D., and Barker, C.F.** Thymus Allografts Fail to Improve Lymphopenia or Prevent Autoimmune Diabetes in the BB Rat. *Transplant. Proceed. 21(1): 218-219, 1989.*

207. **Rossini, A.A., Mordes, J.P., Pelletier, A., and Like, A.A.** Transfusions of Whole Blood Prevent Spontaneous Diabetes Mellitus in the BB/W Rat. *Science 219: 975-977, 1983.*

208. **Rossini, A.A., Faustman, D., Woda, B.A., Like, A.A., Szmanski, I., and Mordes, J.P.** Lymphocyte Transfusions Prevent Diabetes in the Bio-Breeding/Worcester Rat. *J. Clin. Invest. 74: 39-45, 1984.*

209. **Rossini, A.A., Mordes, J.P., Greiner, D.L., Nakano, K., Appel, M.C., and Handler, E.S.** Spleen Cell Transfusion in the Bio-Breeding/Worcester Rat: Prevention of Diabetes, Major Histocompatibility Complex Restriction, and Long-Term Persistence of Transfused Cells. *J. Clin. Invest. 77: 1399-1401, 1986.*

210. **Mordes, J.P., Gallina, D.L., Handler, E.S., Greiner, D.L., Nakamura, N., Pelletier, A., and Rossini, A.A.** Transfusions Enriched for W3/25$^+$ Helper/Inducer T Lymphocytes Prevent Spontaneous Diabetes in the BB/W Rat. *Diabetologia 30: 22-26, 1987.*

211. **Logothetopoulos, J., Shumak, K., and Bailey, D.** Prevention of Spontaneous but not of Adoptively Transferred Diabetes by Injection of Neonatal BB/Hooded Hybrid Rats With Splenocytes or Concanavalin A Blasts from Diabetes-Free Strains. *Diabetes 37(8): 1009-1014, 1988.*

212. **Burstein, D., Mordes, J.P., Greiner, D.L., Stein, D., Nakamura, N., Handler, E.S., and Rossini, A.A.** Prevention of Diabetes in BB/W Rat by Single Transfusion of Spleen Cells: Parameters that Affect Degree of Protection. *Diabetes 38: 24-30, 1989.*

213. **Kuttler, B., Dunger, A., Volk, H.D., Diamantstein, T., and Hahn, H.J.** Prevention and Suppression of Autoimmune Pancreatic Beta-Cell Destruction in BB Rats by Syngeneic Lymphocytes Obtained from Long-Term Normoglycaemic Donors. *Diabetologia 34: 74-77, 1991.*

214. **Like, A.A., Rossini, A.A., Guberski, D.L., Appel, M.C., and Williams, R.M.** Spontaneous Diabetes Mellitus: Reversal and Prevention in the BB/W Rat With Antiserum to Rat Lymphocytes. *Science 206: 1421-1423, 1979.*

215. **Boitard, C., Michie, S., Serrurier, P., Butcher, G.W., Larkins, A.P., and McDevitt, H.O.** *In Vivo* Prevention of Thyroid and pancratic Autoimmunity in the BB Rat by Antibody to Class II Major Histocompatibility Complex Gene Products. *Proc. Nat'l. Acad. Sci. (USA) 82: 6627-6631, 1985.*

216. **Like, A.A., Biron, C.A., Weringer, E.J., Byman, K., Sroczynski, E., and Guberski, D.L.** Prevention of Diabetes in Biobreeding/Worcester Rats With Monoclonal Antibodies that Recognize T Lymphocytes or Natural Killer Cells. *J. Exp. Med. 164: 1145-1159, 1986.*

217. **Markmann, J.F., Jacobson, J.D., Brayman, K.L., Kimura, H., Barker, C.F., and Naji, A.** Prevention of Recurrent Diabetes in BB Rats After Islet Transplantation by Monoclonal Antibody Therapy. *Diabetes 38(Suppl. 1): 165-167, 1989.*

218. **Jacobson, J.D., Markmann, J.F., Brayman, K.L., Barker, C.F., and Naji, A.** Prevention of Recurrent Diabetes in BB Rats by Anti-Asialo-GM1 Antibody. *Diabetes 37(6): 838-841, 1988.*

219. **Barlow, A.K., and Like, A.A.** Anti-CD2 Monoclonal Antibodies Prevent Spontaneous and Adoptive Transfer of Diabetes in the BB/Wor Rat. *Am. J. Pathol. 141(5): 1043-1051, 1992.*

220. **Hahn, H.J., Kuttler, B., Dunger, A., Klöting, I., Lucke, S., Volk, H.D., Baehr, R.V., and Diamantstein, T.** Prolongation of Rat Pancreatic Islet Cell Allograft Survival by Treatment of Recipient Rats With Monoclonal Anti-Interleukin-2-Receptor Antibody and Cyclosporin. *Diabetologia 30: 44-46, 1987.*

221. **Hahn, H.J., Gerdes, J., Lucke, S., Liepe, L., Kauert, C., Volk, H.D., Wacker, H.H., Brocke, S., Stein, H., and Diamantstein, T.** Phenotypical Characterization of the Cells Invading Pancreatic Islets of Diabetic BB/OK Rats: Effect of Interleukin 2 Receptor-Targeted Immunotherapy. *Eur. J. Immunol. 18: 2037-2042, 1988.*

222. **Schreober, S.L., and Crabtree, G.R.** The Mechanism of Action of Cyclosporin A and FK506. *Immunol. Today 13(4): 136-142, 1992.*

223. **Bunjes, D., Hardt, C., Röllinghoff, M., and Wagner, H.** Cyclosporin A Mediates Immunosuppression of Primary Cytotoxic T Cell Responses by Impairing the Release of Interleukin 1 and Interleukin 2. *Eur. J. Immunol. 11: 657-661, 1981.*

224. **Granelli-Piperno, A.** Lymphokine Gene Expression *In Vivo* Is Inhibited by Cyclosporin A. *J. Exp. Med. 171: 533-544, 1990.*

225. **Whitham, R.H., Vandenbark, A.A., Bourdette, D.N., Chou, Y.K., and Offner, H.** Suppressor Cell Regulation of Encephalitogenic T Cell Lines: Generation of Suppressor Macrophages With Cyclosporin A and Myelin Basic Protein. *Cell. Immunol. 126: 290-303, 1990.*

226. **Laupacis, A., Stiller, C.R., Gardell, C., Keown, P., Dupre, J., Wallace, A.C., and Thibert, P.** Cyclosporin Prevents Diabetes in BB Wistar Rats. *The Lancet 1: 10-12, 1983.*

227. **Like, A.A., Anthony, M., Guberski, D.L., and Rossini, A.A.** Spontaneous Diabetes Mellitus in the BB/W Rat: Effects of Glucocorticoids, Cyclosporin-A, and Antiserum to Rat Lymphocytes. *Diabetes 32: 326-330, 1983.*

228. **Stiller, C.R., Laupacis, A., Keown, P.A., Gardell, C., Dupre, J., Thibert, P., and Wall, W.** Cyclosporine: Action, Pharmacokinetics, and Effects in the BB Rat Model. *Metabolism 32 (7 Suppl. 1): 69-72, 1983.*

229. **Like, A.A., Dirodi, V., Thomas, S., Guberski, D.L., and Rossini, A.A.** Prevention of Diabetes Mellitus in the BB/W Rat With Cyclsoporin-A. *Am. J. Pathol. 117: 92-97, 1984.*

230. **Jaworski, M.A., Bayens-Simmonds, J., Rodrigue, J., Mehta, J.G., and Yap, W.** Parameters Associated With the Long-Term Prophylaxis of Diabetes Mellitus in the High-Risk Biobreeding BB Rat. In: *The Immunology of Diabetes.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 367-374.

231. **Yale, J.-F., Grose, M., Seemayer, T.A., and Marliss, E.B.** Immunological and Metabolic Concomitants of Cyclosporin Prevention of Diabetes in BB Rats. *Diabetes 36: 749-757, 1987.*

232. **Brayman, K.L., Armstrong, J., Shaw, L.M., Rosano, T.G., Tomaszewski, J.E., Barker, C.F., and Naji, A.** Prevention of Diabetes in BB Rats by Intermittent Administration of Cyclosporine. *Surgery 102(2): 235-241, 1987.*

233. **Baquerizo, H., Leone, J., Pukel, C., Wood, P., and Rabinovitch, A.** Mechanisms of Cyclosporine Protection Against Spontaneous Diabetes Mellitus in the BB/Wor Rat. *J. Autoimmun. 2: 133-150, 1989.*

234. **Bone, A.J., Walker, R., Varey, A.-M., Cooke, A., and Baird, J.D.** Effect of Cyclosporin on Pancreatic Events and Development of Diabetes in BB/Edinburgh Rats. *Diabetes 39: 508-514, 1990.*

235. **Keenan, R.J., Eiras, G., Burkart, G.J., Stuart, R.S., Hardesty, R.L., Vogelsang, G., Griffith, B.P., and Zeevi, A.** Immunosuppressive Properties of Thalidomide: Inhibition of In Vitro Lymphocyte Proliferation Alone and in Combination with Cyclosporine or FK506. *Transplantation 52(5): 908-910, 1991.*

236. **Shannon, E.J., Ejigu, M., Halle-Mariam, H.S., Berhanm T.Y., and Tasesse, G.** Thalidomide's Effectiveness in Erythema Nodosum Leprosum is Associated with a Decrease in $CD4^+$ Cells in the Peripheral Blood. *Lepr. Rev. 63: 5-11, 1992.*

237. **Neubert, R., Nogueira, A.C., and Neubert, D.** Thalidomide and the Immune System. 2. Changes in Receptors on Blood Cells of a Healthy Volunteer. *Life Sci. 51(26): 2107-2116, 1992.*

238. **Lueprasitisakul, W., Abend, S., Ales, S., Reinhardt, W., Appel, M.C., and Braverman, L.E.** Effect of Thalidomide on the Incidence of Iodine-Induced and Spontaneous Lymphocytic Thyroiditis and Spontaneous Diabetes Mellitus in the BB/Wor Rat. *Acta Endocrinol. (Copenh) 123: 79-83, 1990.*

239. **Sampaio, E.P., Sarno, E.N., Galilly, R., Cohn, Z.A., and Kaplan, G.** Thalidomide Selectively Inhibits Tumor Necrosis Factor α Production by Stimulated Human Monocytes. *J. Exp. Med. 173: 699-703, 1991.*

240. **Moreira, A.L., Sampaio, E.P., Zmuidzinas, A., Frindt, P., Smith, K.A., and Kaplan, G.** Thalidomide Exerts Its Inhibitory Action on Tumor Necrosis Factor α by Enhancing mRNA Degradation. *J. Exp. Med. 177: 1675-1680, 1993.*

241. **Yoon, J.-W., Austin, M., Onodera, T., and Notkins, A.L.** Virus-Induced Diabetes Mellitus: Isolation of a Virus from the Pancreas of a Child with Diabetic Ketoacidosis. *New. Engl. J. Med. 300(21): 1173-1179, 1979.*

242. **Ginsberg-Fellner, F., Witt, M.E., Doberson, M.J., Taub, R., Fedun, B., McEvoy, R.C., Roman, S.H., Davies, T.F., Cooper, L.Z., Rubinstein, T., and Notkins, A.L.** Congenital Rubella Syndrome as a Model for Type I (Insulin-Dependent) Diabetes Mellitus: Increased Prevalence of Islet Cell Surface Antibodies. *Diabetologia 27: 87-89, 1984.*

243. **Leiter, E.H., Fewell, J.W., and Kuff, E.L.** Glucose Induces Intracisternal Type A Retroviral Gene Transcription and Translation in Pancreatic Beta Cells. *J. Exp. Med. 163: 87-100, 1986.*

244. **Onodera, T., Taniguchi, T., Yoshihara, K., Shimizu, S., Sato, M., and Hayashi, T.** Reovirus Type 2-Induced Diabetes in Mice Prevented by Immunosuppression and Thymic Hormone. *Diabetologia 33: 192-196, 1990.*

245. **Yoon, J.-W., and Rayfield, E.J.** Two Possible Mechanisms for Virus-Induced Diabetes. In: *The Immunology of Diabetes Mellitus.* Ed. Jaworski, M.A., et al. Elsevier Science Publishers B.V. 1986. pp 287-298.

246. **Guberski, D.L., Thomas, V.A., Shek, W.R., Like, A.A., Handler, E.S., and Welsh, R.M.** Induction of Type I Diabetes by Kilham's Rat Virus in Diabetes-Resistant BB/Wor Rats. *Science 254: 1010-1013, 1991.*

247. **Schwimmbeck, P.L., Dyrberg, T., and Oldstone, M.B.A.** Abrogation of Diabetes in BB Rats by Acute Virus Infection: Association of Viral-Lymphocyte Interactions. *J. Immunol. 140(10): 3394-3400, 1988.*

248. **Dyrberg, T., Schwimmbeck, P.L., and Oldstone, M.B.A.** The Incidence of Diabetes in BB Rats is Decreased Following Acute LCMV Infection. *Adv. Exp. Med. Biol. 246: 397-402, 1988.*

249. **Southern, P.J., Dyrberg, T., Schwimmbeck, P.L., and Oldstone, M.B.A.** Persistent Virus Infection and Development of Virus-Induced Disease. *J. Autoimmun. 2(Suppl.): 13-20, 1990.*

250. **Campbell, I.L., Hobbs, M.V., Kemper, P., and Oldstone, M.B.A.** Cerebral Expression of Multiple Cytokine Genes in Mice with Lymphocytic Choriomeningitis. *J. Immunol. 152(2): 716-723, 1994.*

251. **Gotfredsen, C.F., Buschard, K., and Frandsen, E.K.** Reduction of Diabetes Incidence of BB Wistar Rats by Early Prophylactic Insulin Treatment of Diabetes-Prone Animals. *Diabetologia 28: 933-935, 1985.*

252. **Vlahos, W.D., Seemayer, T.A., and Yale, J.-F.** Diabetes Prevention in BB Rats by Inhibition of Endogenous Insulin Secretion. *Metabolism 40(8): 825-829, 1991.*

253. **Gottlieb, P.A., Handler, E.S., Appel, M.C., Greiner, D.L., Mordes, J.P., and Rossini, A.A.** Insulin Treatment Prevents Diabetes Mellitus But Not Thyroiditis in RT-6-Depleted Diabetes-Resistant BB/Wor Rats. *Diabetologia 34: 296-300, 1991.*

254. **Bertrand, S., de Paepe, M., Vigeant, C., and Yale, J.-F.** Prevention of Adoptive Transfer in BB Rats by Prophylactic Insulin Treatment. *Diabetes 41: 1263-1277, 1992.*

255. **Koevary, S.B., and Blomberg, M.** Prevention of Diabetes in BB/Wor Rats by Intrathymic Islet Injection. *J. Clin. Invest. 89: 512-516, 1992.*

256. **Posselt, A.M., Barker, C.F., Friedman, A.L., and Naji, A.** Prevention of Autoimmune Diabetes in the BB Rat by Intrathymic Islet Transplantation at Birth. *Science 256: 1321-1324, 1992.*

257. **Rossini, A.A., Slavin, S., Woda, B.A., Geisberg, M., Like, A.A., and Mordes, J.P.** Total Lymphoid Irradiation Prevents Diabetes Mellitus in the Bio-Breeding/Worcester (BB/W) Rat. *Diabetes 33: 543-547, 1984.*

258. **Handler, E.S., Mordes, J.P., McKeever, U., Nakamura, N., Bernhard, J., Greiner, D.L., and Rossini, A.A.** Effects of Irradiation on Diabetes in the BB/Wor Rat. *Autoimmunity 4: 21-30, 1989.*

259. **Hughes, H.P.A., and Babiuk, L.A.** The Adjuvant Potential of Cytokines. *Biotech. Therapeut. 3(3-4): 101-107, 1992.*

260. **Warren, H.S., and Chedid, L.A.** Future Prospects for Vaccine Adjuvants. *CRC Crit. Rev. Immunol. 8(2): 83-101, 1988.*

261. **Toyota, T., Satoh, J., Oya, K., Shintani, S., and Okano, T.** Streptococcal Preparation (OK-432) Inhibits Development of Type I Diabetes in NOD Mice. *Diabetes 35: 496-499, 1986.*

262. **Satoh, J., Shintani, S., Oya, K., Tanaka, S.-I., Nobunga, T., Toyota, T., and Goto, Y.** Treatment With Streptococcal Preparation (OK-432) Suppresses Anti-Islet Autoimmunity and Prevents Diabetes in BB Rats. *Diabetes 37: 1188-1194, 1988.*

263. **Pearce, R.B., and Peterson, C.M.** Studies of Concanavalin A in Nonobese Diabetic Mice. I. Prevention of Insulin-Dependent Diabetes. *J. Pharmacol. Exp. Ther. 258(2): 710-715, 1991.*

264. **Iguchi, M., Inagwa, H., Nishizawa, T., Okutomi, T., Morikawa, A., Soma, G.-I., and Mizuno, D.** Homeostasis as Regulated by Activated Macrophages. V. Suppression of Diabetes Mellitus in Non-Obese Diabetic Mice by LPSw (a Lipopolysaccharide from Wheat Flour). *Chem. Pharm. Bull. 40(4): 1004-1006, 1992.*

265. **Harada, M., Yagi, H., Matsumoto, M., and Makino, S.** Mechanism of Suppressive Action of BCG Against Diabetes in NOD Mice. In: *Frontiers in Diabetes Research. Lessons from Animal Diabetes III.* Ed. Shafrir, E. Smith-Gordon Co. Ltd. 1990. pp 63-66.

266. **Yagi, H., Matsumoto, M., Suzuki, S., Misaki, R., Suzuki, R., Makino, S., and Harada, M.** Possible Mechanism of the Preventative Effect of BCG Against Diabetes Mellitus in NOD Mice. I. Generation of Suppressor Macrophages in Spleen Cells of BCG-Vaccinated Mice. *Cell. Immunol. 138: 130-141, 1991.*

267. **Yagi, H., Matsumoto, M., Kishimoto, Y., Makino, S., and Harada, M.** Possible Mechanism of the Preventative Effect of BCG Against Diabetes Mellitus in NOD Mice. II. Suppression of Pathogenesis by Macrophage Transfer From BCG-Vaccinated Mice. *Cell. Immunol. 138: 142-149, 1991.*

268. **Shintani, S., Satoh, J., Seino, H., Goto, Y., and Toyota, T.** Mechanism of Action of a Streptococcal Preparation (OK-432) In Prevention of Autoimmune Diabetes in NOD Mice: Suppression of Generation of Effector Cells for Pancreatic B Cell Destruction. *J. Immunol. 144(1): 136-141, 1990.*

269. **Seino, H., Satoh, J., Shintani, S., Takahashi, K., Zhu, X.P., Masuda, T., Nobunaga, T., Saito, M., Terano, Y., and Toyota, T.** Inhibition of Autoimmune Diabetes in NOD Mice with Serum from Streptococcal Preparation (OK-432)-Injected Mice. *Clin. Exp. Immunol. 86: 413-418, 1991.*

270. **Satoh, J., Seino, H., Abo, T., Tanaka, S.-I., Shintani, S., Ohta, S., Tamura, K., Sewai, T., Nobunaga, T., Ohteki, T., Kumagai, K., and Toyota, T.** Recombinant Tumor Necrosis Factor α Suppresses Autoimmune Diabetes in Nonobese Diabetic Mice. *J. Clin. Invest. 84: 1345-1348, 1989.*

271. **Kloeting, I., Sadewasser, S., Lucke, S., Vogt, L., and Hahn, J.** Development of BB Rat Diabetes is Delayed or Prevented By Injections or Applications of Immunogens. In: *Frontiers in Diabetes Research. Lessons From Animal Diabetes. II.* Ed. Shafrir, E., and Renold, A.E. John Libbey and Co. Ltd. 1988. pp 190-194.

272. **Sadelain, M.J.W., Qin, H.-Y., Lauzon, J., and Singh, B.** Prevention of Type I Diabetes in NOD Mice by Adjuvant Immunotherapy. *Diabetes 39: 583-589, 1990.*

273. **Sadelain, M.J.W., Qin, H.-Y., Sumoski, W., Parfrey, N., Singh, B., and Rabinovitch. A.** Prevention of Diabetes in the BB Rat by Early Immunotherapy Using Freund's Adjuvant. *J. Autoimmun. 3: 671-680, 1990.*

274. **Qin, H.-Y., Suarez, W.L., Parfrey, N., Power, R.F., and Rabinovitch, A.** Mechanisms of Complete Freund's Adjuvant Protection Against Diabetes in BB Rats: Induction of Non-Specific Suppressor Cells. *Autoimmunity 12: 193-199,* 1992.

275. **McInerney, M.F., Pek, S.B., and Thomas, D.W.** Prevention of Insulitis and Diabetes Onset by Treatment With Complete Freund's Adjuvant in NOD Mice. *Diabetes 40: 715-725, 1991.*

276. **Wang, T., Singh, B., Warnock, G.L., and Rajotte, R.V.** Prevention of Recurrence of IDDM in Islet-Transplanted Diabetic NOD Mice by Adjuvant Immunotherapy. *Diabetes 41: 114-117, 1992.*

277. **Ulaeto, D., Lacy, P.E., Kipnis, D.M., Kanagawa, O., and Unanue, E.R.** A T-Cell Dormant State in the Autoimmune Process of Nonobese Diabetic Mice Treated with Complete Freund's Adjuvant. *Proc. Nat'l. Acad. Sci. (USA) 89: 3927-3931, 1992.*

278. **Shehedeh, N.N., LaRosa, F., and Lafferty, K.J.** Altered Cytokine Activity in Adjuvant Inhibition of Autoimmune Diabetes. *J. Autoimmun. 6: 291-300, 1993.*

279. **Qin, H.-Y., Sadelain, M.J.W., Hitchin, C., Lauzon, J., and Singh, B.** Complete Freund's Adjuvant-Induced T Cells Prevent the Development and Adoptive Transfer of Diabetes in Nonobese Diabetic Mice. *J. Immunol. 150(5): 2072-2080, 1993.*

280. **Serreze, D.V., Hamaguchi, K., and Leiter, E.H.** Immunostimulation Circumvents Diabetes in NOD/Lt Mice. *J. Autoimmun. 2: 759-776, 1989.*

281. **Sobel, D.O., Newsome, J., Ewel, C.H., Bellanti, J.A., Abbassi, V., Creswell, K., and Blair, O.** Poly I:C Induces Development of Diabetes Mellitus in BB Rats. *Diabetes 41: 515-520, 1992.*

282. **Thomas, V.A., Woda, B.A., Handler, E.S., Greiner, D.L., Mordes, J.P., and Rossini, A.A.** Altered Expression of Diabetes in BB/Wor Rats by Exposure to Viral Pathogens. *Diabetes 40: 255-258, 1991.*

283. **Like, A.A., Guberski, D.L., and Butler, L.** Influence of Environmental Viral Agents on Frequency and Tempo of Diabetes Mellitus in BB/Wor Rats. *Diabetes 40: 259-262, 1991.*

284. **Ewel, C.H., Sobel, D.O., Zeligs, B.J., and Bellanti, J.A.** Poly I:C Accelerates Development of Diabetes Mellitus in Diabetes-Prone BB Rats. *Diabetes 41: 1016-1021, 1992.*

285. **Wilson, C.A., Jacobs, C., Baker, P., Baskin, D.G., Dower, S., Lernmark, Å., Toivola, B., Vertrees, S., and Wilson, D.** IL-1β Modulation of Spontaneous Autoimmune Diabetes and Thyroiditis in the BB Rat. *J. Immunol. 144(10): 2784-2788, 1990.*

286. **Vertrees, S., Wilson, S.A., Ubungen, R., Wilson. D., Baskin, D.G., Toivola, B., Jacobs, C., Boiani, N., Baker, P., and Lernmark, Å.** Interleukin-1β Regulation of Islet and Thyroid Autoimmunity in the BB Rat. *J. Autoimmun. 4: 717-732, 1991.*

287. **Kolb, H., Zielasek, J., Treichel, U., Freytag, G., Wrann, M., and Kiesel, U.** Recombinant Interleukin 2 Enhances Spontaneous Insulin-Dependent Diabetes in BB Rats. *Eur. J. Immunol. 16: 209-214, 1990.*

288. **Burstein, D., Handler, E.S., Schindler, J., Seals, J., Mordes, J.P., and Rossini, A.A.** Effect of Interleukin-2 on Diabetes in the BB/Wor Rat. *Diabetes Res. 5: 163-167, 1987.*

289. **Zielasek, J., Burkart, V., Naylor, P., Goldstein, A., Kiesel, U., and Kolb, H.** Interleukin-2-Dependent Control of Disease Development in Spontaneously Diabetic BB Rats. *Immunology 69: 209-214, 1990.*

290. **Allison, J., Malcolm, L., Chosich, N., and Miller, J.F.A.P.** Inflammation But Not Autoimmunity Occurs in Transgenic Mice Expressing Constitutive Levels of Interleukin-2 in Islet β Cells. *Eur. J. Immunol. 22: 1115-1121, 1992.*

291. **Heath, W.R., Allison, J., Hoffmann, M.W., Schönrich, G., Hämmerling, G., Arnold, B., and Miller, J.F.A.P.** Autoimmune Diabetes as a Consequence of Locally Produced Interleukin-2. *Nature 359: 547-549, 1992.*

292. **Foulis, A.K., Farquharson, M.A., and Meager, A.** Immunoreactive α-Interferon in Insulin-Secreting β Cells in Type 1 Diabetes Mellitus. *Lancet ii: 1423-1427, 1987.*

293. **Stewart, T.A., Hultgren, B., Huang, X., Pitts-Meek, S., Hully, J., and MacLachlan, N.J.** Induction of Type I Diabetes by Interferon-α in Transgenic Mice. *Science 260: 1942-1946, 1993.*

294. **Carel, J.-C., Schreiber, R.D., Falqui, L., and Lacy, P.E.** Transforming Growth Factor β Decreases the Immunogenicity of Rat Islet Xenografts (Rat to Mouse) and Prevents Rejection in Association With Treatment of the Recipient With a Monoclonal Antibody to Interferon γ. *Proc. Nat'l. Acad. Sci. (USA) 87: 1591-1595, 1990.*

295. **Nicoletti, F., Meroni, P.L., Landolfo, S., Garigio, M., Guzzardi, S., Barcellini, W., Lunetta, M., Mughini, L., and Zanussi, C.** Prevention of Diabetes in BB/Wor Rats Treated With Monoclonal Antibodies to Interferon-γ. *Lancet 336: 319, 1990.*

296. **Debray-Sachs, M., Carnaoud, C., Boitard, C., Cohen, H., Gresser, I., Bedossa, P., and Bach, J.-F.** Prevention of Diabetes in NOD Mice Treated with Antibody to Murine IFNγ. *J. Autoimmun. 4: 237-248, 1991.*

297. **Kay, T.W.H., Campbell, I.L., Oxbrow, L., and Harrison, L.C.** Overexpression of Class I Major Histocompatibility Complex Accompanies Insulitis in the Non-Obese Diabetic Mouse and is Prevented by Anti-interferon-γ Antibody. *Diabetologia 34: 779-785, 1991.*

298. **Sarvetnick, N., Shizuru, J., Liggit, D., Martin, L., McIntyre, B., Gregory, A., Parslow, T., and Stewart, T.** Loss of Pancreatic Islet Tolerance by β-Cell Expression of Interferon-γ. *Nature 346: 844-847, 1990.*

299. **Campbell, I.L., Kay, T.W.H., Oxbrow, L., and Harrison, L.C.** Essential Role for Interferon-γ and Interleukin-6 in Autoimmune Insulin-Dependent Diabetes in NOD/Wehi Mice. *J. Clin. Invest. 87: 739-742, 1991.*

300. **Campbell, I.L., Oxbrow, L., and Harrison, L.C.** Reduction in Insulitis Following Administration of IFN-γ and TNF-α in the NOD Mouse. *J. Autoimmun. 4: 249-262, 1991.*

301. **Pennline, K.J., Roque-Gaffney, E., and Monahan, M.** Recombinant IL-10 Prevents the Onset of Diabetes in the Nonobese Diabetic Mouse. *Clin. Immunol. Immunopathol. 71(2): 169-175, 1994.*

302. **Stam, W.B., Van Oosterhout, A.J.M., and Nikamp, F.P.** Pharmacologic Modulation of TH1- and TH2-Associated Lymphokine Production. *Life Sci. 53(26): 1921-1934, 1993.*

303. **Scales, W.E., Chensue, S.W., Otterness, I., and Kunkel, S.L.** Regulation of Monokine Gene Expression: Prostaglandin E_2 Suppresses Tumor Necrosis Factor but Not Interleukin-1α or β-mRNA and Cell-Associated Bioactivity. *J. Leukocyte Biol. 45: 416-421, 1989.*

304. **Suzuki, M., Negishi, K., Itabashi, A., Katayama, S., Ishii, J., Komeda, K., and Kawazu, S.** Effects of PGE_1 on the Development of Diabetes and Surface Markers of Lymphocytes in BB/W/Tky Rats. *Diabetes Res. 18: 95-99, 1991.*

305. **Tabata, T., Kinoshita, Y., Fujii, S., Hata, F., Toyokata, T., Kimura, S., and Morii, H.** Protection by Thymosin Fraction 5 From Streptozotocin-Induced Diabetes in Mice. *Cell. Molec. Biol. 35(2): 121-127, 1989.*

306. **Winter, W.E., Robbins, V., Elder, M., Barrett, D., Martin, N., and Maclaren, N.K.** Thymosin and the Spontaneously Diabetic BB Rat. *Autoimmunity 1: 115-123, 1988.*

307. **Watanabe, Y., Inoue, I., Inaba, T., Shimano, H., Gotoda, T., Harada, K., Shimada, M., Kawazu, S., Komeda, K., Yazaki, Y., and Yamada, N.** Effect of Macrophage Colony-Stimulating Factor on the Development of Diabetes Mellitus in BB Rats. *Horm. Metab. Res. 25: 323-324, 1993.*

308. **Dyrberg, T., King, J., Lernmark, Å., and Martin, J.M.** Cortisone Fails to Affect Levels of Islet Cell Surface Antibodies and Incidence of Diabetes in the BB Rat. *Endocrinol. 116(1): 47-50, 1985.*

309. **Smith, C.L., and Hammond, G.L.** An Amino Acid Substitution in BioBreeding Rat Corticosteroid Binding Globulin results in Reduced Steroid Binding Affinity. *J. Biol. Chem. 266(28): 18555-18559, 1991.*

310. **Vilcek, J., and Lee, T.-H.** Tumor Necrosis Actor: New Insights into the Molecular Mechanisms of its Multiple Actions. *J. Biol. Chem. 266(12): 7313-7316, 1991.*

311. **Sherry, B., Jue, D.M., Zentella, A., and Cerami, A.** Characterization of High Molecular Weight Glycosylated Forms of Murine Tumor Necrosis Factor. *Biochem. Biophys. Res. Commun. 173(3): 1072-1078, 1990.*

312. **Csech, K., and Beutler, B.** Alternative Cleavage of the Cachectin/Tumor Necrosis Factor Propeptide in a Larger, Inactive Form of Secreted Protein. *J. Biol. Chem. 264(27): 16256-16260, 1989.*

313. **Keigh, C., Fong, Y., Marano, M.A., Seniuk, S., He, W., Barber, A., Minei, J.P., Felsen, D., Lowry, S.F., and Moldawer, L.L.** Identification of a Novel Tumor Necrosis Factor α/Cachectin From the Livers of Burned and Infected Rats. *Arch. Surg. 125: 79-85, 1990.*

314. **Jue, D.-M., Sherry, B., Luedke, C., Manogue, K.R., and Cerami, A.** Processing of Newly Synthesized Cachectin/Tumor Necrosis Factor in Endotoxin-Stimulated Macrophages. *Biochemistry 29: 8371-8377, 1990.*

315. **Browning, J.L., Androlewicz, M.J., and Ware, C.F.** Lymphotoxin and an Associated 33 kDa Glycoprotein are Expressed on the Surface of an Activated Human T Cell Hybridoma. *J. Immunol. 147(4): 1230-1237, 1991.*

316. **Browning, J.L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E.P., Hession, C., O'Brine-Greco, B., Foley, S.F., and Ware, C.F.** Lymphotoxin β, a Novel Member of the TNF Family that Forms a Heteromeric Complex with Lymphotoxin on the Cell Surface. *Cell 72: 847-856, 1993.*

317. **Lantz, M., Lindvall, L., Pantazis, P., and Olssen, I.** Lymphotoxin Produced by Human B- and T-Cell Lines Appears in Two Distinct Forms. *Molec. Immunol. 28(1/2): 9-16, 1991.*

318. **Vassalli, P.** The Pathophysiology of Tumor Necrosis Factors. *Ann. Rev. Immunol. 10: 411-452, 1992.*

319. **Bonavida, B.** Immunomodulatory Effect of Tumor Necrosis Factor. *Biotherapy 3: 127-133, 1991.*

320. **Gordon, C., and Wolfsy, D.** Effects of Recombinant Murine Tumor Necrosis Factor-α on Immune Function. *J. Immunol. 144(5): 1753-1758, 1990.*

321. **Witsell, A.L., and Schook, L.B.** Tumor Necrosis Factor-α is an Autocrine Growth Regulator During Macrophage Differentiation. *Proc. Nat'l. Acad. Sci. (USA) 89: 4754-4758, 1992.*

322. **Giroir, B.P., Brown, T., and Beutler, B.** Constitutive Synthesis of Tumor Necrosis Factor in the Thymus. *Proc. Nat'l. Acad. Sci. (USA) 89: 4864-4868, 1992.*

323. **Hernández-Casselles, T., and Stutman, O.** Immune Functions of Tumor Necrosis Factor. 1. Tumor Necrosis Factor Induces Apoptosis of Mouse Thymocytes and Can Also Stimulate or Inhibit IL-6-Induced Proliferation Depending on the Concentration of Mitogenic Costimulation. *J. Immunol. 151(8): 3999-4012, 1993.*

324. **Watanabe, Y., and Jacob, C.O.** Regulation of MHC Class II Antigen Expression: Opposing Effects of Tumor Necrosis Factor-α on IFN-γ-Induced HLA-DR and Ia Expression Depends on the Maturation and Differentiation Stage of the Cell. *J. Immunol. 146(3): 899-905, 1991.*

325. **Zimmer, T., and Jones, P.P.** Combined Effects of Tumor Necrosis Factor-α, Prostaglandin E_2, and Corticosterone on Induced Ia Expression on Murine Macrophages. *J. Immunol. 145(4): 1167-1175, 1990.*

326. **Aversa, G., Punnonen, J., and de Vries, J.E.** The 26-kD Transmembrane Form of Tumor Necrosis Factor α on Activated $CD4^+$ T Cell Clones Provides a Costimulatory Signal for Human B Cell Activation. *J. Exp. Med. 177: 1575-1585, 1993.*

327. **Birkland, T.P., Sypek, J.P., and Wyler, D.J.** Soluble TNF and Membrane TNF Expressed on $CD4^+$ Lymphocytes Differ in their Ability to Activate Macrophage Antileishmanial Defense. *J. Leukocyte Biol 51: 296-299, 1992.*

328. **Jacob, C.O., Aiso, S., Michie, S.A., McDevitt, H.O., and Acha-Orbea, H.** Prevention of Diabetes in Nonobese Diabetic Mice by Tumor Necrosis Factor (TNF): Similarities Between TNF-α and Interleukin 1. *Proc. Nat'l. Acad. Sci. (USA) 87: 968-972, 1990.*

329. **Satoh, J., Seino, H., Shintani, S., Tanaka, S.-I., Ohteki, T., Masuda, T., Nobunga, T., and Toyota, T.** Inhibition of Type 1 Diabetes in BB Rats with Recombinant Human Tumor Necrosis Factor-α. *J. Immunol. 145(5): 1395-1399, 1990.*

330. **Gerasimidi, A., Sheehan, K.C.F., Schreiber, R.D., and Lacy, P.E.** Tumor Necrosis Factor-α Prevents Rejection of Islet Xenografts (Rat to Mouse). *Diabetes 42: 651-657, 1993.*

331. **Jacob, C.O., Aiso, S., Schreiber, R.D., and McDevitt, H.O.** Monoclonal Anti-Tumor Necrosis Factor Antibody Renders Non-Obese Diabetic Mice Hypersensitive to Irradiation and Enhances Insulitis Development. *Int. Immunol. 4(5): 611-614, 1992.*

332. **Yang, X.-D., Tisch, R., Cao, Z., Singer, S., Schreiber, R., and McDevitt, H.O.** The Role of Tumor Necrosis Factor-α in the Development of Autoimmunity in NOD Mice. *Autoimmunity 15(Suppl.): 42, 1993.*

333. **Jacob, C.O., Aiso, S., Michie, S.A., McDevitt, H.O., and Acha-Orbea, H.** Inhibition of Adoptive Transfer and Development of Spontaneous Diabetes Mellitus in Nonobese Diabetic Mice by TNF-α. In: *Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy.* Eds. Bonavida, B., and Granger, G. Karger. 1990. pp *222-227.*

334. **Seino, H., Takahashi, K., Satoh, J., Zhu, X.P., Sagara, M., Masuda, T., Nobunaga, T., Funahashi, I., Kajikawa, Toyota, T.** Prevention of Autoimmune Diabetes With Lymphotoxin in NOD Mice. *Diabetes 42: 398-404, 1993.*

335. **Takahashi, K., Satoh, J., Seino, H., Zhu, X.P., Sagara, M., Masuda, T., and Toyota, T.** Prevention of Type I Diabetes With Lymphotoxin in BB Rats. *Clin. Immunol. Immunopathol. 69(3): 318-323, 1993.*

336. **Rothe, H., Fehsel, K., and Kolb, H.** Tumour Necrosis Factor Alpha Production is Upregulated in Diabetes Prone BB Rats. *Diabetologia 33: 573-575, 1990.*

337. **Rothe, H., Öngören, C., Rösen, P., and Kolb, H.** Genetic Analysis of Aberrant Tumor Necrosis Factor α Production in Prediabetic BB Rats. *Transplant. Proceed. 25(5): 2833-2834, 1993.*

338. **Rothe, H., Öngören, C., Martin, S., Rösen, P., and Kolb, H.** Abnormal TNF-α Production in Diabetes-Prone BB Rats: Enhanced TNF-α Expression and Defective PGE_2 Feedback Inhibition. *Immunology 81: 407-413, 1994.*

339. **Janjic, D., Asfari, M., and Piguet, J.F.** Tumor Necrosis Factor α (TNF) Production in the BB Rat. *Diabetologia 33(Suppl.): A193, 1990.*

340. **Tanaka, S.-I., Seino, H., Satoh, J., Fujii, N., Rikiishi, H., Zhu, X.P., Takahashi, K., Sagara, M., Nobunaga, T., and Toyota, T.** Increased *In Vivo* Production of tumor Necrosis Factor After Development of Diabetes in Nontreated, Long-Term Diabetic BB Rats. *Clin. Immunol. Immunopathol. 62(3): 258-263, 1992.*

341. **Kirisits, M.J., Vardimon, D., Kunz, H.W., and Gill III, T.J.** Mapping of the *TNFA* Locus in the Rat. *Immunogenetics 39: 59-60, 1994.*

342. **Jacob, C.O., and Hwang, F.** Definition of Microsatellite Size Variants for *Tnfa* and *Hsp70* in Autoimmune and Nonautoimmune Mouse Strains. *Immunogenetics 36: 182-188, 1992.*

343. **Pociat, F., Briant, L., Jongeneel, C.V., Mölvig, J., Worsaae, H., Abbal, M., Thomsen, M., Nerup, J., and Cambon-Thomsen, A.** Association of Tumor Necrosis Factor (TNF) and Class II Major Histocompatibility Complex Alleles with the Secretion of TNF-α and TNF-β by Human Mononuclear Cells: A Possible Link to Insulin-Dependent Diabetes Mellitus. *Eur. J. Immunol. 23: 224-231, 1993.*

344. **Pociat, F., Wilson, A.G., Nerup, J., and Duff, G.W.** No Independent Association Between a Tumor Necrosis Factor-α Promoter Region Polymorphism and Insulin-Dependent Diabetes Mellitus. *Eur. J. Immunol. 23: 3043-3049, 1993.*

345. **Higuchi, Y., Herrera, P., Muniesa, P., Huarte, J., Belin, D., Ohashi, P., Aichele, P., Orci, L., Vassalli, J.-D., and Vassalli, P.** Expression of a Tumor Necrosis Factor α Transgene in Murine Pancreatic β Cells Results in Severe and Permanent Insulitis Without Evolution Towards Diabetes. *J. Exp. Med. 176: 1719-1731, 1992.*

346. **Picarella, D.E., Kratz, A., Li, C.-B., Ruddle, N.H., and Flavell, R.A.** Insulitis in Transgenic Mice Expressing Tumor Necrosis Factor β (Lymphotoxin) in the Pancreas. *Proc. Nat'l. Acad. Sci. (USA) 89: 10036-10040, 1992.*

347. **Picarella, D.E., Kratz, A., Li, C.-B., Ruddle, N.H., and Flavell, R.A.** Transgenic Tumor Necrosis Factor (TNF)-α Production in Pancreatic Islets Leads to Insulitis, Not Diabetes. *J. Immunol. 150(9): 4136-4150, 1993.*

348. **Ohashi, P.S., Oehen, S., Aichele, P., Pircher, H., Odermatt, B., Herrera, P., Higuchi, Y., Buerki, K., Hengartner, H., and Zinkernagel, R.M.** Induction of Diabetes is Influenced by the Infectious Virus and Local Expression of MHC Class I and Tumor Necrosis Factor-α. *J. Immunol. 150(11): 5185-5194, 1993.*

349. **Lapchak, P.H.** Cellular Immune Function in the Diabetes-Prone BB Rat: Effector Mechanisms in Islet Cell Cytotoxicity. *Master of Science Thesis, Department of Pathology, McGill University, 1988.*

350. **Wu, G., Field, C.J., and Marliss, E.B.** Glucose and Glutamine Metabolism in Rat Macrophages: Enhanced Glycolysis and Unaltered Glutaminolysis in Spontaneously Diabetic BB Rats. *Biochem. Biophys. Acta. 115: 166-173, 1991.*

351. **Branch, D.R., Shah, A., and Guilbert, L.J.** A Specific and Reliable Bioassay for the Detection of Femtomolar Levels of Human and Murine Tumor Necrosis Factors. *J. Immunol. Methods. 143: 251-261, 1991.*

352. **Hiernaux, J., Bona, C., and Barker, P.J.** Neonatal Treatment with Low Doses of Anti-Idiotypic Antibody Leads to the Expression of a Silent Clone. *J. Exp. Med. 153: 1004-1008, 1981.*

353. **Waters, C.A., Pilarski, L.M., Wegmann, T.G., and Diener, E. I.** Presence of Active Suppression n Mice Rendered Tolerant to Human γ-Globulin *In Utero* Correlates with the Breakdown of the Tolerant State. *J. Exp. Med. 149: 1134-1151, 1979.*

354. **Dresser, D.W.** Chapter 8: immunization of Experimental Animals. In: *Handbook of Experimental Immunology, Volume 1, Immunochemistry.* Ed. Weir, D.M. Blackwell Scientific Publications. 1986 pp 8.1-8.21.

355. **Gorevic, P.D., Prelli, F.C., and Frangione, B.** Immunoglobulin G (IgG). *Meth. Enzymol. 116: 1-25, 1985.*

356. **Karkar, A.M., Koshino, Y., Cashman, S.J., Dash, A.C., Bonnefoy, J., Meager, A., and Rees, A.J.** Passive Immunization Against Tumour Necrosis Factor-Alpha (TNF-α) and IL-1β Protects from LPS Enhancing Glomerular Injury in Nephrotoxic Nephritis in Rats. *Clin. Exp. Immunol. 90: 312-318, 1992.*

357. **Olsen, D.M., Lye, S.J., Skinner, K., and Challis, J.R.G.** Early Changes in Prostaglandin Concentrations in Ovine Maternal and Fetal Plasma, Amniotic Fluid and From Dispersed Cells of Intrauterine Tissues Before the Onset of ACTH-Induced Pre-Term Labour. *J. Reprod. Fert. 71: 45-55, 1984.*

358. **Harvey, S., Merry, B.J., and Phillips, J.G.** Influence of Stress on the Secretion of Corticosterone in the Duck (*Anas Platyrynchos*). *J. Endocrinol. 87: 161-171, 1980.*

359. **Sunderland, C.A., McMaster, W.R., and Williams, A.F.** Purification with Monoclonal Antibody of a Predominant Leukocyte-Common Antigen and Glycoprotein from Rat Thymocytes. *Eur. J. Immunol. 9: 155-159, 1979.*

360. **Robinson, A.P., White, T.M., and Mason, D.W.** MRC OX-43: A Monoclonal Antibody which Reacts with all Vascular Endothelium in the Rat Except that of Brain Capillaries. *Immunology 57: 231-237, 1986.*

361. **Fukumoto, T., McMaster, W.R., and Williams, A.F.** Mouse Monoclonal Antibodies Against Rat Major Histocompatibility Antigens. Two Ia Antigens and Expression of Ia and Class I Antigens in Rat Thymus. *Eur. J. Immunol. 12: 237-243, 1982.*

362. **Galley, P., Jongeneel, C.V., Barras, C., Burnier, M., Baumgartner, J.-D., Glauser, M.P., and Heumann, D.** Short Time Exposure to Lipopolysaccharide is Sufficient to Activate Human Monocytes. *J. Immunol. 150(11): 5086-5093, 1993.*

363. **Girard, R., Pedron, T., and Chaby, R.** Endotoxin-Induced Expression of Endotoxin Binding Sites on Murine Bone Marrow Cells. *J. Immunol. 150(10): 4504-4513, 1993.*

364. **Jacob, C.O., and McDevitt, H.O.** Tumour Necrosis Factor-α in Murine Autoimmune "Lupus" Nephritis. *Nature 331: 356-358, 1988.*

365. **Gordon, C., Ranges, G.E., Greenspan, J.S., and Wofsy, D.** Chronic Therapy with Recombinant Tumor Necrosis Factor-α in Autoimmune NZB/NZW F_1 Mice. *Clin. Immunol. Immunopathol. 52: 421-434, 1989.*

366. **Wanidworanun, C., and Strober, W.** Predominant Role of Tumor Necrosis Factor-α in Human Monocyte IL-10 Synthesis. *J. Immunol. 151(12): 6853-6861, 1993.*

367. **Held, W., MacDonald, H.R., Weissman, I.L., Hess, M.W., and Mueller, C.** Genes Encoding Tumor Necrosis Factor α and Granzyme A are Expressed During Development of Autoimmune Diabetes. *Proc. Nat'l. Acad. Sci. (USA) 87: 2239-2243, 1990.*

368. **Anderson, J.T., Cornelius, J.G., Jarpe, A.J., Winter, W.E., and Peck, A.B.** Insulin-Dependent Diabetes in the NOD Mouse Model II. β Cell Destruction in Autoimmune Diabetes is a T_{h2} and Not a T_{h1} Mediated Event. *Autoimmunity 15: 113-122, 1993.*

369. **Del Prete, G.F., Tiri, A., De Cario, M., Mariotti, S., Pinchera, A., Chretien, I., Romagnani, S., and Ricci, M.** High Potential to Tumor Necrosis Factor α (TNF-α) Production of Thyroid Infiltrating T Lymphocytes in Hashimoto's Thyroiditis: A Peculiar Feature of Destructive Thyroid Autoimmunity. *Autoimmunity 4: 267-276, 1989.*

370. **Turner, M., Londi, M., and Feldmann, M.** Human T Cells from Autoimmune and Normal Individuals can Produce Tumor Necrosis Factor. *Eur. J. Immunol. 17: 1807-1814, 1987.*

371. **Murray, L.J., Lee, R., and Martens, C.** *In Vivo* Cytokine Gene Expression in T Cell Subsets of the Autoimmune MRL/Mp-*lpr/lpr* Mouse. *Eur. J. Immunol. 20: 163-170, 1990.*

372. **Davidson, W.F., Calkins, C., Hügin, A., Giese, T., and Holmes, K.L.** Cytokine Secretion by C3H-*lps* and -*gld* T Cells. Hypersecretion of IFN-γ and Tumor Necrosis Factor-α by Stimulated CD4+ T Cells. *J. Immunol. 146(12): 4138-4148, 1991.*

373. **Broome Powell, M., Mitchell, D., Lederman, J., Buckmeier, J., Zamvil, S.S., Graham, M., Ruddle, N.H., and Steinman, L.** Lymphotoxin and Tumor Necrosis Factor-Alpha Production by Myelin Basic Protein-Specific T Cell Clones Correlates with Encephalitogenicity. *Int. Immunol. 2(6): 539-544, 1990.*

374. **Kinkhabwala, M., Sehajpal, P., Skolnik, E., Smith, D., Sharma, V.K., Vlassara, H., Cerami, A., and Suthanthiran, M.** A Novel Addition to the T Cell Repertory. Cell Surface Expression of Tumor Necrosis Factor/Cachectin by Activated Normal T Cells. *J. Exp. Med. 171: 941-946, 1990.*

375. **Rothe, J., Gehr, G., Loetscher, H., and Lessiauer, W.** Tumor Necrosis Factor Receptors-Structure and Function. *Immunol. Res. 11: 81-90, 1992.*

376. **Crowe, P.D., VanArsdale, T.L., Walter, B.N., Ware, C.F., Hession, C., Ehrenfels, B., Browning, J.L., Din, W.S., Goodwin, R.G., and Smith, C.A.** A Lymphotoxin-β-Specific Receptor. *Science 264: 707-710, 1994.*

377. **Ranges, G.E., Zlotnik, A., Espevik, T., Dinarello, C.A., Cerami, A., and Palladino Jr., M.A.** Tumor Necrosis Factor α/Cachectin is a growth Factor for Thymocytes. Synergistic Interactions with Other Cytokines. *J. Exp. Med. 167: 1472-1478, 1988.*

378. **Suda, T., and Zlotnik, A.** *In Vitro* Induction of CD8 Expression on Thymic Pre-T Cells. I. Transforming Growth Factor-β and Tumor Necrosis Factor-α Induce CD8 Expression on $CD8^-$ Thymic Subsets Including the $CD25^+CD3^-CD4^-CD8^-$ Pre-T Cell Subset. *J. Immunol. 148(6): 1737-1745, 1992.*

379. **Suda, T., and Zlotnik, A.** *In Vitro* Induction of CD8 Expression on Thymic Pre-T Cells. II. Characterization of $CD3^-CD4^-CD8\alpha^+$ Cells Generated *In Vitro* by Culturing $CD25^+CD3^-CD4^-CD8^-$ Thymocytes with T Cell Growth Factor-β and Tumor Necrosis Factor-α. *J. Immunol. 149(1): 71-76, 1992.*

380. **Muñoz-Fernández, M.A., Pimentel-Muiños, F.X., González, A., Gambon, F., Alvarez-Vallina, L., and Fresno, M.** Differential Effect of Tumor Necrosis Factor on Human Thymocyte Subpopulations. *Immunology 76: 439-445, 1992.*

381. **Mordes, J.P., Desemone, J., and Rossini, A.A.** The BB Rat. *Diabetes/Metabolism Rev. 3(3): 725-750, 1987.*

382. **Rossini, A.A., Handler, E.S., Greiner, D.L., and Mordes, J.P.** Insulin Dependent Diabetes Mellitus. Hypothesis of Autoimmunity. *Autoimmunity 8: 221-235, 1991.*

383. **Imura, H., Fukata, J-I., and Mori, T.** Cytokines and Endocrine Function: An Interaction Between the Immune and Neuroendocrine Systems. *Clin. Endocrinol 35: 107-115, 1991.*

384. **Derijk, R., and Berkenbosch, F.** The Immune-Hypothalamo-Pituitary-Adrenal Axis and Autoimmunity. *Intern. J. Neuroscience 59: 91-100, 1991.*

385. **Sternberg, E.M., Young III, W.S., Bernardini, R., Calogero, A.E., Chrousos, G.P., Gold, P.W., and Wilder, R.L.** A Central Nervous System Defect in Biosynthesis of Corticotropin-Releasing Hormone is Associated with Susceptibility to Streptococcal Cell Wall-Induced Arthritis in Lewis Rats. *Proc. Nat'l. Acad. Sci. (USA) 86: 4771-4775, 1989.*

386. **Sternberg, E.M., Hill, J.M., Chrousos, G.P., Kamilaris, T., Listwack, S.J., Gold, P.W., and Wilder, R.L.** Inflammatory Mediator-Induced Hypothalamic-Pituitary-Adrenal Axis Activation is Defective in Streptococcal Cell Wall Arthritis-Susceptible Lewis Rats. *Proc. Nat'l. Acad. Sci. (USA) 86: 2374-2378, 1989.*

387. **Kroemer, G., Brezinschek, H.-P., faessler, R., Schauenstein, K., and Wick, G.** Physiology and Pathology of an Immunoendocrine Feedback Loop. *Immunol. Today 9(6): 163-165, 1988.*

388. **Salkowski, C.A., and Vogel, S.N.** Lipopolysaccharide Increases Glucocorticoid Receptor Expression in Murine Macrophages. A Possible Mechanism for Glucocorticoid-Mediated Suppression of Endotoxicity. *J. Immunol. 149(12): 4041-4047, 1992.*

389. **Carter, W.R., Herrman, J., Stokes, K., and Cox, D.J.** Promotion of Diabetes Onset by Stress in the BB Rat. *Diabetologia 30: 674-675, 1987.*

390. **Weingarten, R., Skiar, L.A., Mathison, J.C., Omidi, S., Ainsworth, T., Simon, S., Ulevitch, R.J., and Tobias, P.S.** Interactions of Lipopolysaccharide with Neutrophils in Blood Via CD14. *J. Leukocyte Biol. 53: 518-524, 1993.*

391. **Lei, M.-G., and Morrison, D.C.** Specific Endotoxic Lipopolysaccharide-Binding Proteins on Murine Splenocytes. I. Detection of Lipopolysaccharide-Binding Sites on Splenocytes and Splenocyte Subpopulations. *J. Immunol. 141(3): 996-1005, 1988.*

392. **Pugin, J., Ulevitch, R.J., and Tobias, P.S.** A Critical Role for Monocytes and CD14 in Endotoxin-Induced Endothelial Cell Activation. *J. Exp. Med. 178: 2193-2200, 1993.*

393. **Zhang, Z., and Morrison, D.C.** Lipopolysaccharide Structure-Function Relationship in Activation Versus Reprogramming of Mouse Peritoneal Macrophages. *J. Leukocyte Biol. 54: 444-450, 1993.*

394. **Morrison, D.C., Lei, M.-G., Kirikae, T., and Chen, T.-Y.** Endotoxin Receptors on Mammalian Cells. *Immunobiol. 187: 212-226, 1993.*

395. **Dentener, M.A., Bažil, V., Von Asmuth, E.J.U., Ceska, M., and Buurman, W.A.** Involvement of CD14 in Lipopolysaccharide-Induced Tumor Necrosis Factor-α, IL-6, and IL-8 Release by Human Monocytes and Alveolar Macrophages. *J. Immunol. 150 (7): 2885-2891, 1993.*

396. **Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C.** CD14, a Receptor for Complexes of Lipopolysaccharide (LPS) and LPS Binding Protein. *Science 249: 1431-1433, 1990.*

397. **Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S., and Ulevitch, R.J.** Structure and Function of Lipopolysaccharide Binding Protein. *Science 249: 1429-1431, 1990.*

398. **Ziegler-Heitbrock, H.W.L., and Ulevitch, R.J.** CD14: Cell Surface Receptor and Differentiation Marker. *Immunol. Today 14(3): 121-125, 1993.*

399. **Economou, J.S., McBride, W.H., Essner, R., Rhoades, K., Golub, S., Holmes, E.C., and Morton, D.L.** Tumour Necrosis Factor Production by IL-2-Activated Macrophages *In Vitro* and *In Vivo. Immunology 67: 514-519, 1989.*

400. **Narumi, S., Finke, J.H., and Hamilton, T.A.** Interferon γ and Interleukin 2 Synergize to Induce Selective Monokine Expression in Murine Peritoneal Macrophages. *J. Biol. Chem. 265(12): 7036-7041, 1990.*

401. **Dong, Z., Lu, S., and Zhang, Y.** Effects of Pretreatment with Protein Kinase C Activators on Macrophage Activation for Tumor Cytotoxicity, Secretion of Tumor Necrosis Factor and its mRNA Expression. *Immunobiol. 179: 382-394, 1989.*

402. **Celada, A., and Maki, R.A.** IFN-γ Induces the Expression of the Genes for MHC Class II I-Aβ and Tumor Necrosis Factor Through a Protein Kinase C-Independent Pathway. *J. Immunol. 146(1): 114-120, 1991.*

403. **Hart, P.H., Cooper, R.L., and Finley-Jones, J.J.** IL-4 Suppresses IL-1β, TNF-α and PGE_2 Production by Human Peritoneal Macrophages. *Immunology 72: 344-349, 1991.*

404. **Oswald, I.P., Wynn, T.A., Sher, A., and James, S.L.** Interleukin 10 Inhibits Macrophage Microbicidal Activity by Blocking the Endogenous Production of Tumor Necrosis Factor α Required as a Costimulatory Factor for Interferon γ-Induced Activation. *Proc. Nat'l. Acad. Sci. (USA) 89: 8676-8680, 1992.*

405. **Minty, A., Chalon, P., Derocq, J.-M., Dumont, X., Guillemot, J.-C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P., and Caput, D.** Interleukin-13 is a New Human Lymphokine Regulating Inflammatory and Immune Responses. *Nature 363: 248-250, 1993.*

406. **Kunkel, S.L., Wiggins, R.C., Chensue, S.W., and Larrick, J.** Regulation of Macrophage Tumor Necrosis Factor Production by Prostaglandin E_2. *Biochem. Biophys. Res. Commun. 137(1): 404-410, 1986.*

407. **Letari, O., Nicosia, S., Chiavaroli, C., Vacher, P., and Schlegel, W.** Activation by Bacterial Lipopolysaccharide Causes Changes in the Cytosolic Free Calcium Concentration in Single Peritoneal Macrophages. *J. Immunol. 147(3): 980-983, 1991.*

408. **Chen, T.-Y., Lei, M.-G., Suzuki, T., and Morrison, D.C.** Lipopolysaccharide Receptors and Signal Transduction Pathways in Mononuclear Phagocytes. *Curr. Topics Microbiol. Immunol. 181: 169-188, 1992.*

409. **Weinstein, S.L., June, C.H., and Defrance, A.L.** Lipopolysaccharide-Induced Protein Tyrosine Phosphorylation in Human Macrophages is Mediated by CD14. *J. Immunol. 151(7): 3829-3838, 1993.*

410. **Renz, H., Gong, J.-H., Schmidt, A., Nain, M., and Gemsa, D.** Release of Tumor Necrosis Factor-α from Macrophages: Enhancement and Suppression Are Dose-Dependently Regulated by Prostaglandin E_2 and Cyclic Nucleotides. *J. Immunol. 141(7): 2388-2393, 1988.*

411. **Lehmmann, V., Benninghoff, B., and Dröge, W.** Tumor Necrosis Factor-Induced Activation of Peritoneal Macrophages is Regulated by Prostaglandin E_2 and cAMP. *J. Immunol. 141(2): 587-591, 1988.*

412. **Müller, J.M., Lőms Zeigler-Heitbrock, H.W., and Baeurele, P.A.** Nuclear Factor kappa B, a Mediator of Lipopolysaccharide Effects. *Immunobiol. 187: 233-256, 1993.*

413. **Unanue, E.R., and Cerottini, J.-C.** Antigen Presentation. *FASEB J. 3: 2496-2502, 1989.*

414. **Pistoresi-Palencia, M.C., Romero-Piffiguer, M., and Ferro, M.E.** Effects of Aging on the Autoimmune Response to Rat Male Accessory Glands: Deficit of I-E Positive Peritoneal Cells Capable of Inducing Suppression. *Eur. J. Immunol. 21: 1685-1689, 1991.*

415. **Offner, H., Brostoff, S.W., and Vandenbark, A.A.** Antibodies Against I-A and I-E Determinants Inhibits the Activation and Function of Encephalitogenic T-Lymphocyte Lines. *Cell. Immunol. 100: 364-373, 1986.*

416. **Faustman, D., Li, X., Lin, H.Y., Fu, Y., Eisenbarth, G., Avruch, J., and Guo, J.** Linkage of Faulty Major Histocompatibility Complex Class I to Autoimmune Diabetes. *Science 254: 1756-1761, 1991.*

417. **Neta, R., Sayers, T.J., and Oppenheim, J.J.** Chapter 22. Relationship of TNF to Interleukins. In: *Tumor Necrosis Factors. Structure, Function, and Mechanism of Action.* Eds. Aggarwal, B.B., and Vilcek, J. Marcel Dekker, Inc. 1992. pp 499-566.

418. **Bonavida, B.** Chapter 4. TNF as Immunomodulatory Agent. In: *Tumor Necrosis Factors. Structure, Function, and Mechanism of Action.* Eds. Aggarwal, B.B., and Vilcek, J. Marcel Dekker, Inc. 1992. pp 315-329.

419. **Rutherford, M.S., and Schook, L.B.** Macrophage Function in Response to PGE_2, L-Arginine Deprivation, and Activation by Colony-Stimulating Factors is Dependent on Hematopoietic Stimulus. *J. Leukocyte Biol. 52: 228-235, 1992.*

420. **Alleva, D.G., Burger, C.J., and Elgert, K.D.** Tumor-Induced Macrophage Tumor Necrosis Factor-α Production Suppresses Autoreactive T Cell Proliferation. *Immunobiol. 188: 430-445, 1993.*

421. **Alleva, D.G., Askew, D., Burger, C.J., and Elgert, K.D.** Fibrosarcoma-Induced Increase in Macrophage Tumor Necrosis Factor α Synthesis Suppresses T Cell Responses. *J. Leukocyte Biol. 54: 152-160, 1993.*

422. **Walker, T.M., Yurochko, A.D., Burger, C.J., and Elgert, K.D.** Ia$^-$ Macrophages and Cytokine Networks Contribute to Tumor-Induced Suppression of CD4$^+$ Autoreactive T Cells. *Immunol. Invest. 22(3): 169-187, 1993.*

423. **Phipps, R.P., Stein, S.H., and Roper, R.L.** A New View of Prostaglandin E Regulation of the Immune Response. *Immunol. Today 12(10): 349-352, 1991.*

424. **Betz, M., and Fox, B.S.** Prostaglandin E_2 Inhibits Production of Th1 Lymphokines but Not of Th2 Lymphokines. *J. Immunol. 146(1): 108-113, 1991.*

425. **Muñoz, E., Beutner, U., Zubiaga, A.M., and Huber, B.T.** IL-1 Activates Two Separate Signal Transduction Pathways in T Helper Type II Cells. *J. Immunol. 144(3): 964-969, 1990.*

426. **Muñoz, E., Beutner, U., Zubiaga, A.M., Merrow, M., Sauter, N.P., and Huber, B.T.** Cholera Toxin Discriminates Between T Helper 1 and 2 Cells in T Cell Receptor-Mediated Activation: Role of cAMP in T Cell Proliferation. *J. Exp. Med. 172: 95-103, 1990.*

427. **Pfizenmaier, K., Himmler, A., Schütze, S., Scheurich, P., and Krönke, M.** Chapter 28. TNF Receptors and TNF Signal Transduction. In: *Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine.* Ed. Beutler, B. Raven Press. 1992. pp 439-472.

428. **Kruppa, G., Thoma, B., Machleidt, T., Wiegmann, K., and Krönke, M.** Inhibition of Tumor Necrosis Factor (TNF)-Mediated NF-kB Activation by Selective Blockade of the Human 55-kDa TNF Receptor. *J. Immunol. 148(10): 3152-3157, 1992.*

429. **Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendly, B.M., and Palladino Jr., M.A.** Stimulation of Human T-Cell Proliferation by Specific Activation of the 75-kDa Tumor Necrosis Factor Receptor. *J. Immunol. 151(9): 4637-4641, 1993.*

430. **Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino Jr., M.A., and Goeddel, D.V.** The Two Different Receptors for Tumor Necrosis Factor Mediate Distinct Cellular Responses. *Proc. Nat'l. Acad. Sci. (USA) 88: 9292-9296, 1991.*

431. **Barbara, J.A.J., Smith, W.B., Gamble, J.R., Van Ostade, X., Vandenabeele, P., Tavernier, J., Fiers, W., Vadas, M.A., and Lopez, A.F.** Dissociation of TNF-α Cytotoxic and Proinflammatory Activities by p55 Receptor- and p75 Receptor-Selective TNF-α Mutants. *EMBO J. 13(4): 843-850, 1994.*